Aspects of the glucose metabolism of Trypanosoma brucei

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Award date:
1987

Awarding institution:
University of Bath

ASPECTS OF THE GLUCOSE METABOLISM OF *TRYPANOSOMA BRUCEI*

submitted by Alison Panes

for the degree of PhD

of the University of Bath

1987

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Acknowledgements

My thanks to Dr. Robert Eisenthal for his help and encouragement throughout this work and to all the members of the Biochemistry Department at the University of Bath for making my time there both useful and enjoyable.

I am grateful to the University of Bath for their funding of this studentship.

Abbreviations

The abbreviations used in this thesis are those recommended by the Biochemical Society ((1984), 217, 1 - 26) except:

α-GP	α-glycerophosphate
α-GPDH	α-glycerophosphate dehydrogenase
α-GPO	α-glycerophosphate oxidase
DHAP	Dihydroxyacetone phosphate
T(G)	Ringer-phosphate buffer containing 10mM glucose
T(SG)	Ringer-phosphate buffer containing 10mM glucose and sucrose at a concentration of 3g/100ml
PEG	Polyethylene glycol
RPMI medium 1640	Roswell Park Memorial Institute medium, formulation 1640.

Summary

The transition between the aerobic and anaerobic pathways of glucose metabolism in Trypanosoma brucei was investigated. It was found that the anaerobic pathway is inoperative under the oxygen tensions of the mammalian bloodstream but may operate in the cerebrospinal fluid or at the site of inflammation.

A scheme was devised for the purification of the enzyme α-glycerophosphate dehydrogenase (α-GPDH, EC 1.1.1.8) from T. brucei and kinetic studies carried out upon the pure enzyme extract. It was established that the enzyme has a compulsory-order mechanism with NADH binding to the enzyme first and NAD^+ being released last. Kinetic constants of the enzyme in the absence and in the presence of product inhibitors were determined. Calculations show that in the presence of the products α-GP and NAD^+ at the concentrations purported to exist in the glycosome, α-GPDH is able to maintain the overall glycolytic flux in T. brucei.

Contents

CHAPTER 1: INTRODUCTION

1.1: The medical and economic importance of Trypanosoma brucei

Perhaps one of the best-studied parasitic protozoa of the last decade has been, indeed still is, the salivarian trypanosome Trypanosoma brucei brucei, and it is this organism on which the work reported here has been carried out. Since T. b. brucei is a causative agent only of nagana, a bovine disease found primarily in Africa, such intense interest may seem inexplicable until it is realised that T. b. brucei is both morphologically and biochemically indistinguishable from several other members of the subgenus Trypanozoon (Hoare, 1964). These other members, Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense are the trypanosomes which in man cause acute and chronic African sleeping sickness respectively. A component of human serum, thought to be a high-density lipoprotein (Rifkin, 1978), brings about the lysis of T. b. brucei but not T. b. rhodesiense or T. b. gambiense and the former is therefore preferred for safe experimental work. During this work, the term Trypanosoma brucei will be used to refer to the members of the T. b. brucei-rhodesiense-gambiense complex collectively.

Trypanosoma brucei brucei is in fact an extremely important parasite in its own right, nagana denying the use of up to ten million square kilometres of grazing land in Africa. This land could support at least 140 million head of cattle while only 20 million are actually grazed there (Molyneux and Ashford, 1983) and of these over three million die each year (Fairlamb, 1982). Extra cattle production would

not only yield food supplies but would also increase the nitrogenous content of the soil and make available more draught animals. Human suffering in the affected areas is exacerbated by the severe shortage of both meat and dairy produce, resulting in malnutrition and increased susceptibility to diseases, not least among which are the human trypanosomiases.

1.2: The classification of trypanosomes and the diseases they cause

Trypanosomes may be classified as shown in Figure 1 and the genus *Trypanosoma* conveniently further subdivided into salivarian and stercorarian trypanosomes according to the mode of transmission by the insect vector. The most important stercorarian trypanosome is the South American species *T. cruzi*, the causative agent of Chagas' disease. Typical of stercorarian trypanosomes, *T. cruzi* divides in the hind-gut of the vector, usually the reduviid (triatomid) bug, and then enters the human host in the faeces of the vector through abrasions on the skin or *via* the internal mucosa following ingestion. Although the incidence of Chagas' disease is restricted to Central and South America the World Health Authority has estimated that in 1980 some 35 million people were exposed to infection and between 13 and 14 million actually infected (Molyneux and Ashford, 1983). Even untreated, Chagas' disease is not inevitably fatal although young children often die during the initial febrile stages (Fairlamb, 1982), while adults are more likely to survive to the non-symptomatic latent stage which may last indefinitely. Up to 20% of patients will develop the final, chronic phase of the disease however, when trypanosomal

Figure 1: The classification of Trypanosoma brucei (after Cox, 1982; Molyneux and Ashford, 1983)

KINGDOM ; Animalia

SUB-KINGDOM ; Protozoa

PHYLUM ; Sarcomastigophora

CLASS ; Zoomastigophora

ORDER ; Kinetoplastida

GENUS ; Trypanosoma

SECTION ; Salivaria

SUB-GENUS ; Trypanozoon

SPECIES ; Trypanosoma brucei brucei

Trypanosoma brucei rhodesiense

Trypanosoma brucei gambiense

for example

antigens attach to the surface of both infected and uninfected cells, particularly those of the endocardium, stimulating attack by the immune system of the host. The initial interaction between parasite and the target cells of the host cardiovascular system may be due to the removal of sialic acid from these cells by a neuraminidase of trypanosomal origin (Libby et al., 1986). The disease has then become autoimmune and 95% of patients possess an antibody which reacts with human heart muscle and leads to the most common cause of death from Chagas' disease, congestive heart failure (Boreham, 1979). There are no satisfactory drugs currently available for prophylactic use against Chagas' disease although two, a 5-nitrofuran derivative (Nifurtimox, Bayer 2502) and a 2-nitroimidazole (Benznidazole, Radanil) are used to treat both acute and chronic phases (Gutteridge, 1985). These drugs have such severe side-effects however that the full recommended course is rarely completed. The development of more acceptable alternatives has been hampered by the discovery that the most promising nitro-compounds show inherent carcinogenic and mutagenic properties (Molyneux and Ashford, 1983). A vaccine against T. cruzi is by no means an impossibility and research continues into all aspects of both the prevention and treatment of this important disease.

That this research unfortunately cannot be applied to the human African trypanosomiases, known collectively as African sleeping sickness, is due to the fact that they bear little resemblance to Chagas' disease in any respect other than the genus of their causative agents. The protozoa which cause African sleeping sickness are members of the subgenus Trypanozoon and are classified as salivarian as they develop in the proboscis or salivary glands of the insect

vector, being then transmitted by an inoculative method during feeding (Smyth, 1976). In contrast to the South American trypanosomiasis, African sleeping sickness is invariably fatal if untreated although the time to death varies. Infection with T. b. gambiense gives rise to a chronic form of the disease, suffered primarily by the inhabitants of riverine areas of West Africa (Figure 2), in which the time to death may be between nine months and three years or more (Donaldson, 1979). Infection by T. b. rhodesiense, occurring mainly in the savannah of Africa , gives rise to the more acute form of the disease which may prove fatal within several months (Meshnick, 1984). Some 45 million people are exposed to the risk of infection in Africa and of these between ten and 20 thousand new cases are recorded annually (Goodwin, 1985).

The distinction between the two forms of African sleeping sickness is often difficult to make. In cases of chronic African sleeping sickness (caused by T. b. gambiense) pathological changes occur at the site of injection of the parasite, in the bloodstream, in the extravascular spaces of the lymphatic and connective tissues and of certain visceral organs including the heart and, in the later stages, in the central nervous system (CNS) particularly the brain and spinal cord. The pathology of acute African sleeping sickness is very similar but the lymphatic glands are less commonly involved and death often occurs before the CNS involvement is as advanced as in the chronic form of the disease. In the acute form however, the lesions of the heart are more frequent and more severe than in the chronic form (Donaldson, 1979). The actual means by which trypanosomiasis causes death is still uncertain (Markell and Voge, 1981) but it is

Figure 2: The geographical distribution of the occurrence of sleeping sickness in Africa. The solid dots indicate areas of T. b. rhodesiense endemicity. The open areas are T. b. gambiense foci which have a higher level of endemicity and from which epidemics arise (Molyneux and Ashford, 1983)

known that the parasites have an immunosuppressive action often leading to secondary infections such as pneumonia, a frequent cause of death (Molyneux and Ashford, 1983).

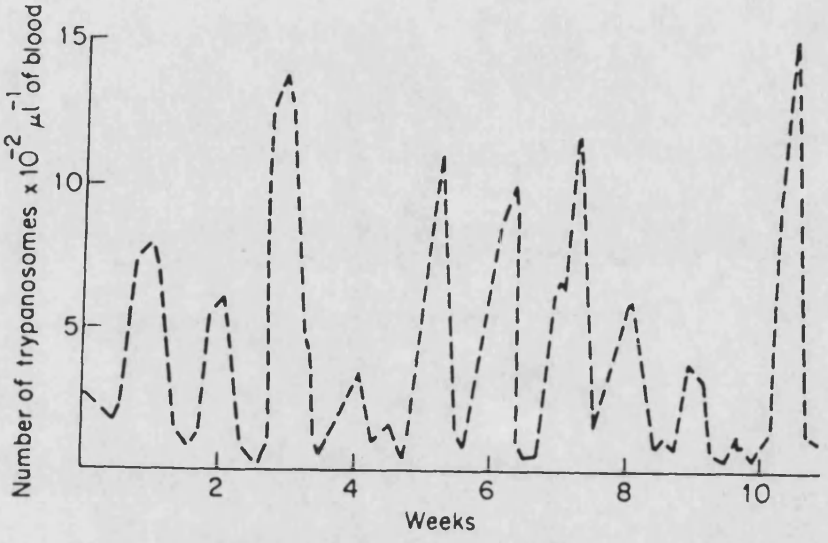
Chronic African sleeping sickness results from the bite of an infected tsetse fly usually of the Glossina palpalis group, and is thought to have a man-fly-man cycle, while acute African sleeping sickness is usually transmitted by tsetse flies of the G. mortisans group and a man-fly-man cycle of infection is rare, certain game animals being reservoirs of T. b. rhodesiense (Markell and Voge, 1981).

1.3: The variable surface antigen

The diagnosis of both forms of the disease is often hampered by a characteristic fluctuating parasitaemia which means that parasites are occasionally not detectable in the peripheral blood in the early stages of the disease (Figure 3a). This cyclic parasitaemia is due to the ability of trypanosomes of the brucei subgroup to undergo antigenic variation, a result of the presence of the so-called variable surface antigen (VSA) (Whitfield,1979). The bloodstream forms of T. brucei are completely enveloped in a monomolecular glycoprotein coat, 12 to 15nm thick (Vickerman, 1969) and comprising up to ten million molecules per cell (Whitfield, 1979), produced while the parasite occupies the salivary glands of the tsetse vector (Vickerman et al., 1980) (Figure 4). When the trypanosomes are injected by the fly into the host the majority possess glycoprotein coats of a single kind, the homotype. A minority of this population

Figure 3a: The fluctuating parasitaemia in the blood of a patient with African sleeping sickness (Wakelin, 1984)

Figure 3b: The role of antigenic variation in the relapsing infection (Chappell, 1980)


Number of trypanosomes x 10^{-2} μl^{-1} of blood
15
10
5
2
4
6
8
10
Weeks

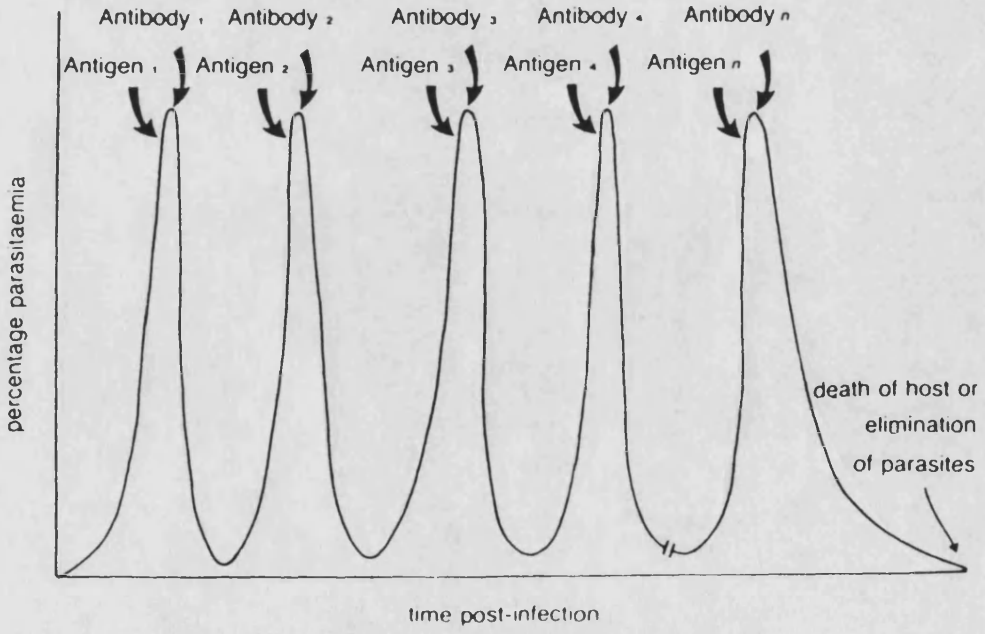
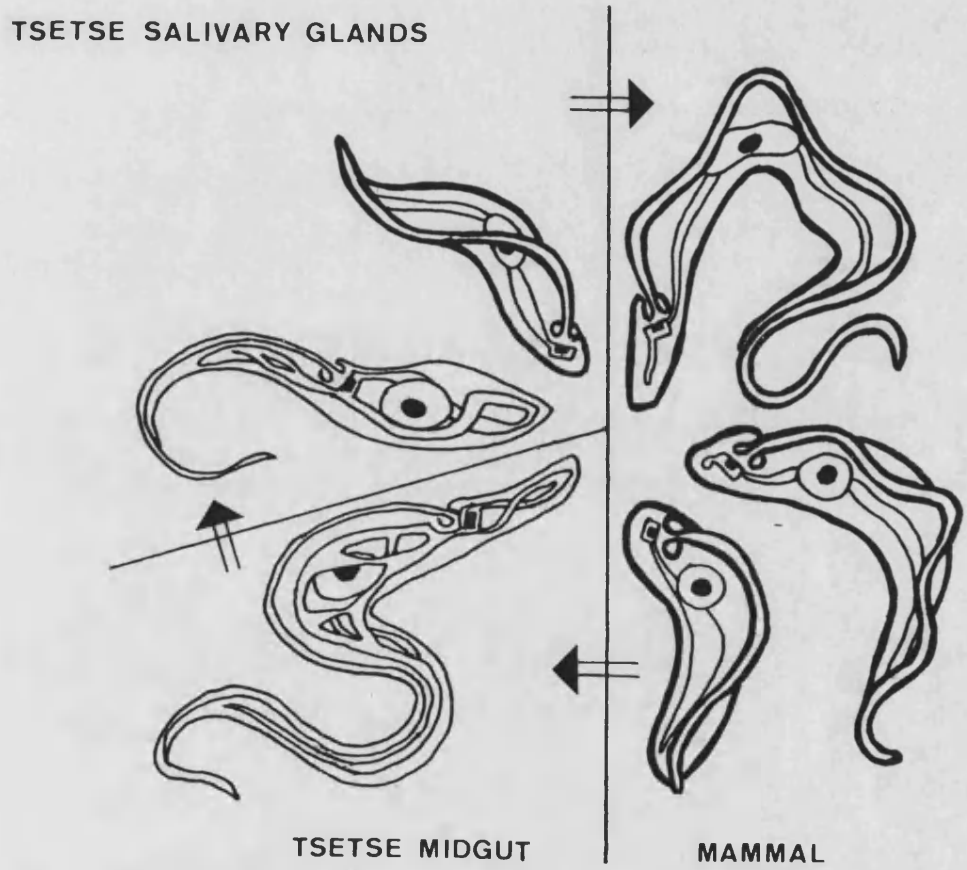

Antibody 1
Antibody 2
Antibody 3
Antibody 4
Antibody n
Antigen 1
Antigen 2
Antigen 3
Antigen 4
Antigen n
percentage parasitaemia
death of host or
elimination
of parasites
time post-infection

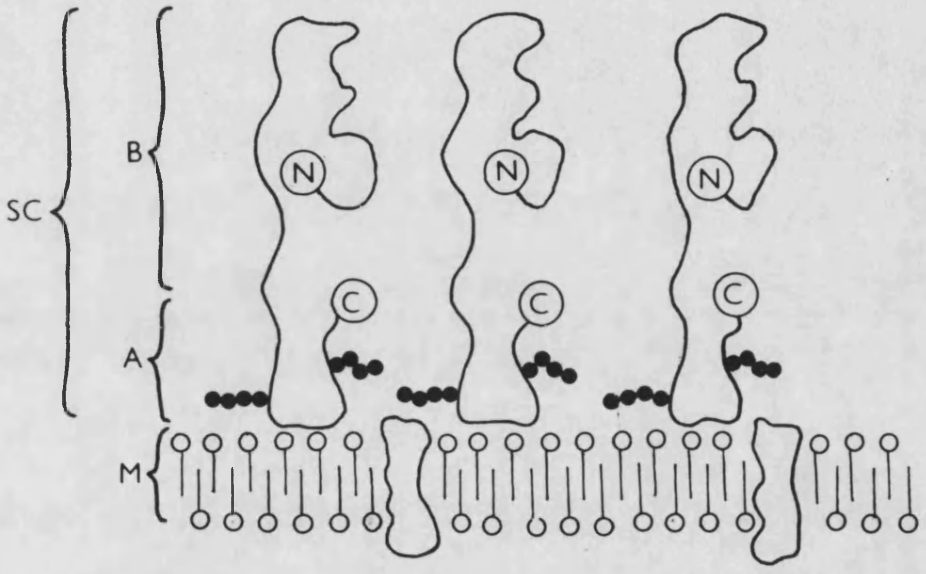
Figure 4: Stages in the life-cycle of Trypanosoma brucei showing the presence or absence of a surface coat. Forms which possess a surface coat are drawn with a thick outline (after Vickerman, 1969)


TSETSE SALIVARY GLANDS
TSETSE MIDGUT
MAMMAL

however display variations in the polypeptide part of the glycoprotein giving rise to antigenically-different coats or heterotypes. The immune system of the host quickly responds by raising IgM-type antibodies to the homotype (Poltera 1985; Vickerman, 1985) and the parasites are destroyed by agglutination and complement-mediated lysis (Cox, 1982). The parasites which possess heterotypical VSA's are not destroyed however and survive, those with the most common heterotypical VSA to become the next homotype and so this cycle is repeated (Figure 3a). As new heterotypes are constantly being produced the parasite population is continually one step ahead of the immune system of the host (Cox, 1982). It has recently been estimated that a single organism possesses between several hundred and a thousand genes encoding for the VSA, between five and ten per cent of the total genetic capacity of the trypanosome (Donelson and Turner, 1985). Since the discovery of the trypanosomal VSA much research has been carried out into its structure and genetic determination in the hope that eventually a vaccine against African sleeping sickness may be developed, possibly using some constant part of the VSA molecule as antigen.

As yet no such results have been obtained although the structure of the VSA has been elucidated (Figure 5). The VSA is a glycoprotein with an approximate molecular weight of 65,000 (Cross and Johnson, 1976). Each molecule consists of a single polypeptide chain of approximately 600 amino acid residues (Mansfield, 1981) with there being probably no more than 50% homology at the C-terminal and little or none at the extremely variable N-terminal. There may be some amino acid homology in the inner segment of the molecule, where a common

Figure 5: Structural model of the surface coat (SC) of Trypanosoma brucei. The glycoprotein molecules are shown attached to the outer surface of the membrane (M). Each molecule has a C-terminal domain (A) which contains most of the carbohydrate (●●●) and a somewhat conservative strucure. The outer N-terminal domain (B) is highly-variable in amino acid sequence (Whitfield, 1979).


SC
B
A
M
N
N
N
C
C
C

structure may be anticipated for the region concerned with attachment of the coat molecule to the trypanosomal plasma membrane or in regions governing the three-dimensional arrangement of the molecule (Cross and Johnson, 1976). The means of attachment of the glycoprotein molecule to the plasma membrane has recently been elucidated by Ferguson et al. (1985), who suggest that the carboxyl-terminal of the polypeptide is covalently-linked to glycosyl-sn-1,2-dimyristylphosphatidylinositol in the membrane, and that the release of the coat is catalysed by an endogeneous phospholipase C.

Another potentially important discovery has been that the metacyclic form of trypanosome, the form injected by the vector into the host, shows on its surface initially only a limited variety of variable surface antigens, perhaps a maximum of 15 (Donelson and Turner, 1985). The antigens expressed by the so-called metacyclic genes are expressed for only a short time after infection, approximately five days, after which these particular genes are "switched-off" and the infection continues with the normal wide range of antigens. Currently the only possibility for vaccination against the African trypanosomiases seems to be against the limited number of variable surface antigens which are expressed at the beginning of an infection, although it has been suggested that should these antigens be suppressed another set would soon replace them (Kolata, 1984).

1.4: Control of the tsetse vector

Thus far then, and certainly not in the short-term future, the spread of African sleeping sickness cannot be controlled by

vaccination and currently the most effective method of control of trypanosomiasis is the avoidance of the tsetse fly by one of two means (Molyneux and Ashford, 1983). The first, the movement of human populations to tsetse-free areas, is expensive, disruptive and inconvenient and any success is quickly reversed by the breakdown of surveillance and diagnostic facilities brought about by frequent civil unrest in the African countries most at risk (Goodwin, 1985). The second method of reducing tsetse fly-man contact is obviously by control of the size and distribution of the fly population. This latter method depends on a variety of techniques including alteration of the habitat, pesticide application, genetic and biological control, traps and physiological interference. Alteration of the habitat by clearing of the bush or shooting reservoir game animals is unacceptable in the interests of conservation and is therefore no longer in common use (Ukoli, 1984). Genetic control of tsetse populations is by the release of male insects, sterilised by chemicals or irradiation, in sufficiently large numbers to swamp the natural population of wild males (Jordan, 1985). This is an expensive process which may only prove useful when used in conjunction with one or more other methods of control; although it has been used successfully to eradicate the screwworm (Cochliomyia hominivorax) in the south eastern United States, similar success has not been attained in the south western United States or northern Mexico where the influx of new flies from the more southerly parts of Mexico has made complete control impossible. Similar difficulties are easy to envisage in Africa (Molyneux, 1982). Biological control, by means of vector pathogens, parasites or predators (Jordan, 1985) and physiological interference

by means of insect growth regulators which inhibit chitin synthesis (Molyneux, 1982) are both the subjects of continuing research and ongoing field-trials. The use of traps of various types has become the subject of much interest with the development of the biconical trap. The objective of the traps is to attract tsetse flies to a lethal target, a trap impregnated with an insecticide such as deltamethrin, rather than to release the insecticide into the general environment.

Until recently the attraction of the traps has been solely on a visual basis, but it has been discovered that the use of powerful olfactory attractants can enhance the catch of a trap by up to twenty times (Jordan, 1985). The first attractant to be identified was a component of ox breath, but the obvious disadvantages of its use have led to the current research into the production of a comparable synthetic compound for use in biconical traps. Should a suitable odour be found, the estimate that deltamethrin-impregnated biconical traps could reduce Glossina populations in riverine areas by over 99% (Laveissiere and Couret, 1980) may easily become a reality. The incidence of nagana could be reduced by the exploitation of the trypanotolerance shown by some breeds of cattle. These cattle, primarily humpless taurine breeds, are smaller than the more commonly-kept zebu and not such prolific producers of milk. It remains for long-term breeding programmes to combine the most desirable characteristics of the various breeds (Molyneux and Ashford, 1983).

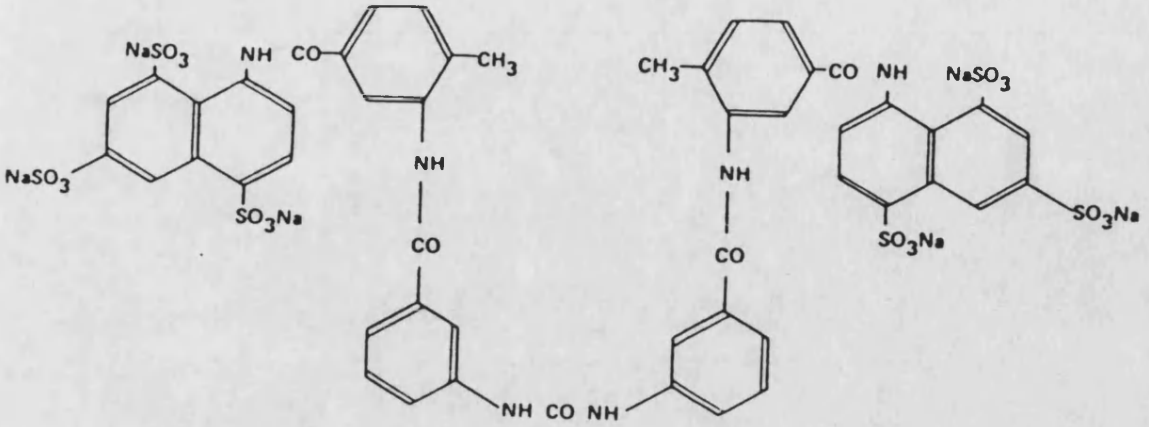
1.5: Anti-trypanosomal drugs currently in use

Whilst these methods of vector elimination are under investigation the control of African sleeping sickness falls to the use of such drugs as there are. The three key drugs currently in use are those listed as essential trypanocidal drugs by the World Health Organisation in 1983. These drugs, all introduced before 1955 (Howells, 1985), are pentamidine for chemoprophylaxis, suramin for treatment of the early stages of the disease and melarsoprol for the later stages when there is CNS involvement (Figure 6). As yet there is no drug in routine use for the prevention of transmission of trypanosomes during blood transfusion.

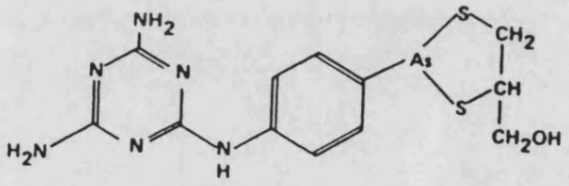
Pentamidine, a highly positively-charged aromatic diamidine, is the preferred agent for the prophylaxis of African sleeping sickness and can also be used therapeutically against the early stages of the chronic, and to a lesser extent the acute, form of the disease. It is unable to cross the blood-brain barrier and therefore ineffective in the later stages when there is CNS involvement. The selectivity of the drug between host and parasite seems to be due to the presence in the parasite of a high-affinity, energy-requiring transport system (Damper and Patton, 1976) which raises the intracellular concentration of pentamidine to many times the concentration in the plasma. The mode of action of pentamidine is not yet fully resolved but seems to exert its effect on the synthesis of deoxyribonucleic acid (DNA), probably binding to DNA by a non-intercalative mechanism by virtue of its positive charge at physiological pH, with the synthesis of the uniquely-trypanosomal kinetoplast DNA appearing to be particularly

Figure 6: The structures of the anti-trypanosomal drugs most commonly-used in the treatment of African sleeping sickness. Structure a is that of Suramin, structure b represents melarsoprol and structure c is pentamidine (Gutteridge, 1985).

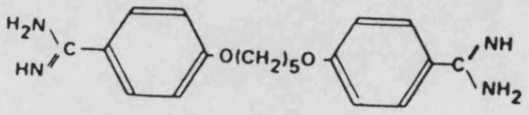
A


NaSO3
NH
CO
CH3
NaSO3
SO3Na
NH
CO
NH CO NH
CH3
CO NH
NaSO3
NH
CO
SO3Na
SO3Na

B


NH2
N
N
H2N
N
N
H
As
S
CH2
S
CH
CH2OH

C


H2N
C
HN
O(CH2)5O
C
NH
NH2

sensitive to disruption (Gutteridge, 1985). The course of treatment is short, the most serious side-effect when pentamidine is given intramuscularly being nephrotoxicity (Meshnik, 1984), although more commonly histamine is released leading to temporary hypotension and faintness (Gutteridge, 1985). Some parasite resistance to pentamidine is now becoming apparent (Rée, 1985) but it is the inability of the drug to transverse the blood-brain barrier which limits its usefulness.

The highly-polar sulphated-naphthylamine suramin is similarly unable to penetrate into the cerebrospinal fluid (Meshnik, 1984) and therefore can only be used in the early stages of either form of the disease. The drug is administered to the patient intravenously and slowly taken up by the trypanosome by endocytosis in the form of a plasma-protein-bound complex (Gutteridge, 1985). Once the drug is within the cell the rates of respiration and glycolysis are both progressively reduced (Fairlamb and Bowman, 1980b), suramin being a potent inhibitor, competitive with respect to α-glycerophosphate, of both enzymes involved in the reoxidation of NADH by the so-called α-glycerophosphate shuttle (see Figure 7). Whilst one of these enzymes, α-glycerophosphate oxidase, is found in the trypanosome but not the mammalian host, the other, NAD^+-linked α-glycerophosphate dehydrogenase, is found in both trypanosome and mammalian cells although in a less important role in the latter (Gutteridge, 1985). The selectivity of suramin is consequently not entirely reliable. Apart from the inability of suramin to pass into the cerebrospinal fluid, the drug has a further disadvantage in its many side-effects which range in severity from immediate nausea and collapse to

blindness and renal failure, often fatal.

The third trypanocidal drug in common use is melarsoprol, an aromatic arsenical which can rapidly cure all stages of both the acute and chronic forms of African sleeping sickness. In homogenates of trypanosomes melarsoprol has been shown to inhibit several enzymes of carbohydrate metabolism including pyruvate kinase (Flynn and Bowman, 1974), glycerol kinase (Hammond and Bowman, 1980b) and the mitochondrial α-glycerophosphate oxidase (Fairlamb and Bowman, 1977). Trivalent arsenicals have a high affinity for the sulphydryl groups which form part of the active site of many enzymes, particularly kinases, and Flynn and Bowman (1974) have shown that, in intact trypanosomes, pyruvate kinase is particularly affected with a consequent intracellular accumulation of phosphoenolpyruvate. Pyruvate kinase is the enzyme most affected in vivo as it is located in the cytoplasm of the trypanosome while the other enzymes mentioned are contained within a membrane-bound microbody-like organelle, the glycosome (Fairlamb, 1982). The absence of any energy reserves in the trypanosome, the slight degree of preferential binding to trypanosomal pyruvate kinase over host pyruvate kinase and some concentration of arsenical drugs in the trypanosome combine to give melarsoprol a measure of selectivity (Gutteridge, 1985). For all its obvious usefulness, melarsoprol has severe side-effects; up to 18% of patients suffer reactive encephalopathy, which occasionally proves fatal, and more commonly fever and pain in the chest or abdomen. For this reason it is recommended that melarsoprol is administered only under hospital supervision and reactions monitored for up to ten days (Molyneux and Ashford, 1983; Gutteridge, 1985).

Figure 7: Pathways of glycolysis in bloodstream-form trypanosomes. The reactions enclosed in the broken box are assumed present under anaerobic conditions. The enzymes indicated are:

E1 - triose phosphate isomerase

E2 - α-GPDH

E3 - glycerol kinase

E4 - α-GPO

Other abbreviations include:

G6P - glucose-6-phosphate

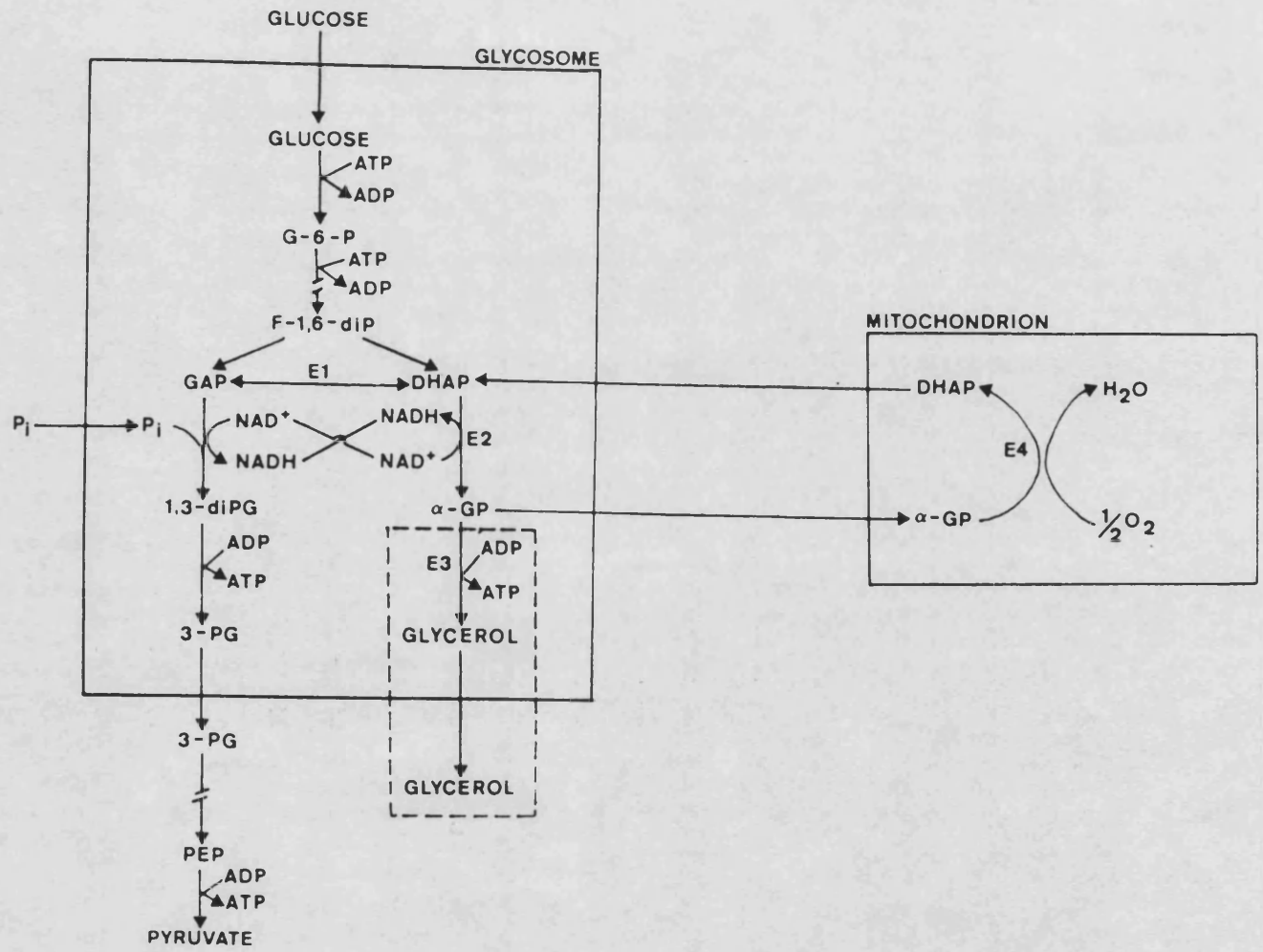
F16diP - fructose-1,6-diphosphate

GAP - glyceraldehyde-3-phosphate

1,3-diPG - 1,3-diphosphoglycerate

3-PG - 3-phosphogluconate

PEP - phosphoenolpyruvate


GLUCOSE
GLYCOSOME
GLUCOSE
ATP
ADP
G-6-P
ATP
ADP
F-1,6-diP
GAP
E1
DHAP
Pi
Pi
NAD+
NADH
NADH
NAD+
E2
1,3-diPG
α-GP
ADP
ATP
E3
ADP
ATP
3-PG
GLYCEROL
3-PG
GLYCEROL
PEP
ADP
ATP
PYRUVATE
MITOCHONDRION
DHAP
H2O
E4
α-GP
½O2

It is immediately apparent from this brief description of the three most beneficial drugs currently in use that further research into anti-trypanosomal chemotherapy is long overdue. In many Third World countries up to 40% of the total annual health-care budget may be spent on drugs; the available drugs should therefore be effective, safe and inexpensive. However the development and screening of new drugs is necessarily time-consuming and costly. A "vicious circle" ensues whereby the people who most need the new drug are unable to purchase it and the resultant poor market forces the price to be maintained at an elevated level (Goodwin, 1985; Rée, 1985). The potential return on any investment in this field by a pharmaceutical company is therefore likely to be less than the outlay, by no means an attractive commercial proposition. The research into the biochemistry and molecular biology of trypanosomes currently being carried out in academic institutions worldwide remains the most promising aspect of the search for new drugs. The discovery of a reaction unique to the trypanosome, an enzyme with markedly different properties to that of the host or a means of vaccination could lead to the development of a valuable life-saving drug.

1.6: The morphology and life-cycle of Trypanosoma brucei

The unusual morphology and life-cycle of the members of the brucei subgroup of trypanosomes has already been the subject of much research, and the ultrastructure of a "typical" member is shown as Figure 8. The plasma membrane of the trypanosome is a typical unit membrane, similar in appearance to that of other animal cells, beneath

Figure 8: The ultrastructure of a typical member of the subgenus Trypanozoon (after Vickerman and Cox, 1967). The long, slender forms are approximately 30μm in length and short, stumpy forms are between 14 and 20μm long. The features labelled are:

A - fold of pellicle

B - attached flagellum

(A and B together form the 'undulating membrane')

C - microtubules of pellicle shown at anterior end only

D - mitochondrion

E - endoplasmic reticulum

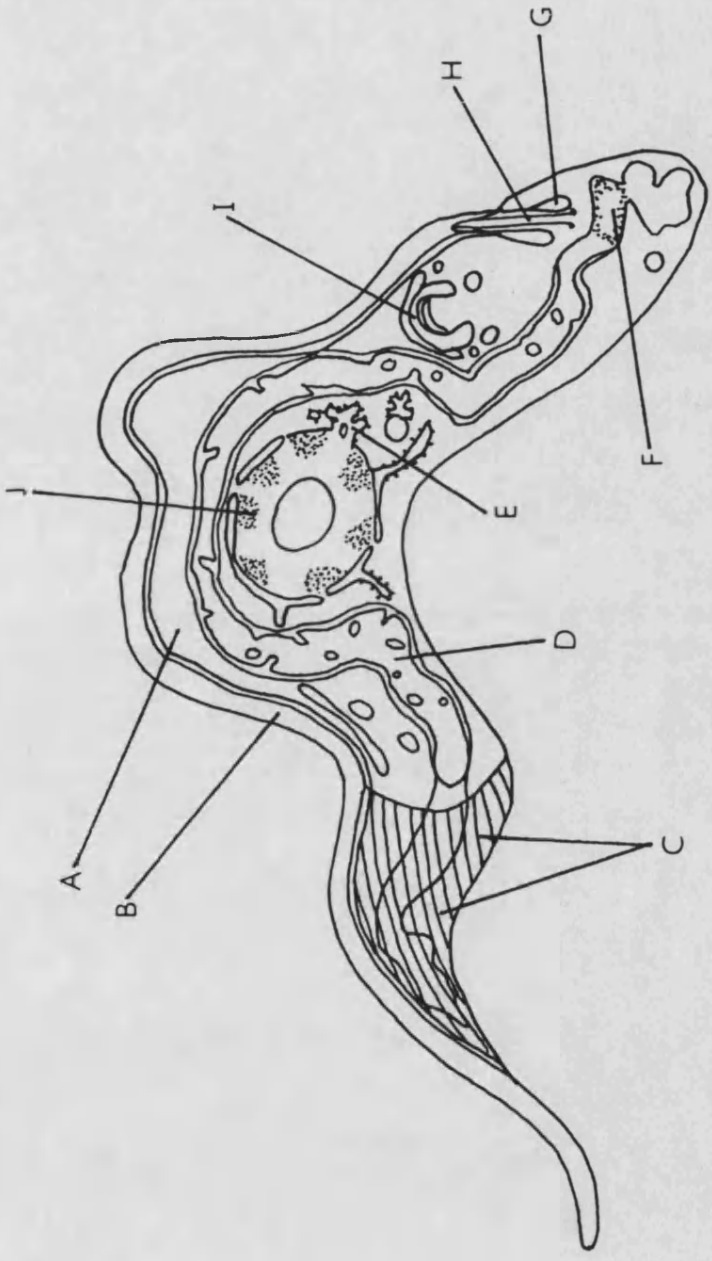
F - kinetoplast

G - reservoir

H - basal body of flagellum

I - golgi apparatus

J - nucleus


A
B
C
D
E
F
G
H
I
J

which lies a regular array of pellicular microtubules, cytoskeletal in function and present in both the vertebrate and invertebrate forms of the parasite (Molyneux and Ashford, 1983). The so-called "undulating membrane" seen under the light microscope may actually be an optical artefact; ultrastructural studies suggest that the flagellar membrane and the plasma membrane are attached by desmosome-like structures, sliding of the flagellar microtubules seeming to cause the plasma membrane to undulate (Smyth, 1976). Trypanosomes are classified in the order Kinetoplastida as a consequence of the presence of the characteristic kinetoplast, an organelle located within the single mitochondrion. The kinetoplast is a complex network of mitochondrial DNA which has been visualised by electron microscopy as thousands of interlocked circular molecules, representing up to 30% of the total cellular DNA (Opperdoes, 1985). One component of the kDNA network, the maxicircle, has features similar to conventional mitochondrial DNA and is present at between 25 and 50 copies per network, each copy being identical in nucleotide sequence. The major transcripts of maxicircle DNA are ribosomal and messenger RNA molecules (Fairlamb, 1982) and there is also evidence that the maxicircles contain the genes for the cytochrome oxidase subunits I, II and III, apocytochrome b. However the second component of the network in T. brucei, the minicircles, of which there are approximately six thousand, has a heterogeneous nucleotide sequence but as yet no known function (Opperdoes, 1985).

An unusual feature of African trypanosomes is their possession of a single mitochondrion in which striking changes are seen during the various stages of the life-cycle. In the insect stages, the

mitochondrion is fully-developed with numerous plate-like cristae, while the mammalian forms have simple, unbranched mitochondria with few, tubular cristae (Fairlamb, 1982) (Figure 9). It is the degree of development of the mitochondrion coupled with the change in shape of the organism, the position of the flagellum as determined by its starting point (Hoare and Wallace, 1966) and the position of the kinetoplast relative to the nucleus (Vickerman, 1985) which combine to describe the morphological stages of the life-cycle (Figure 10). When an infected tsetse fly bites an uninfected animal or human, trypanosomes are injected into the skin in the metacyclic form. At the site of the bite, these metacyclic forms transform into bloodstream forms which then multiply to form a characteristic chancre. The trypomastigotes migrate to the lymph nodes and the bloodstream where they multiply and invade the intracellular spaces of certain tissues (van Meirvenne and Le Ray, 1985). In a typical chronic relapsing infection bloodstream trypomastigotes display a heterogeneity of morphology known as pleomorphism (Markell and Voge, 1981). The long, slender form is numerous in the rising parasitaemia stage of the cycle (Figure 3) while the short, stumpy form is usually seen during the remission phase (Molyneux and Ashford, 1983). After an uninfected tsetse fly has fed on an infected animal the short, stumpy forms which have been ingested transform in the insect midgut to procyclic trypomastigote forms which, after multiplication, migrate to the insect salivary glands. In the salivary glands the procyclic forms transform first into epimastigote forms and then once again into the infective metacyclic form (Vickerman, 1985). Multiplication of trypanosomes has thus far been believed to be by means of binary

Figure 9: Two examples of stages in the life-cycle of Trypanozoon species, showing the position of the extranuclear DNA (the kinetoplast) and the extent of development of the mitochondrion (Newton, 1968).

a) Epimastigote (insect salivary glands)

b) Trypomastigote (mammalian bloodstream)

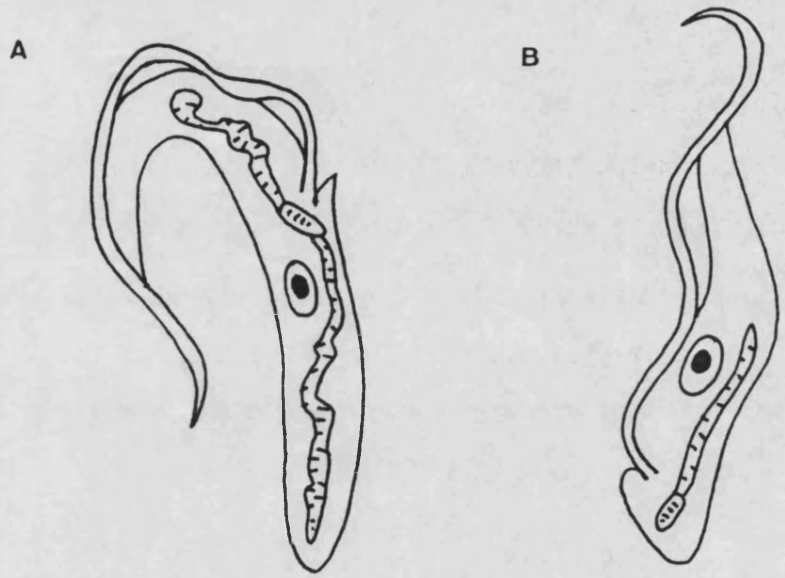
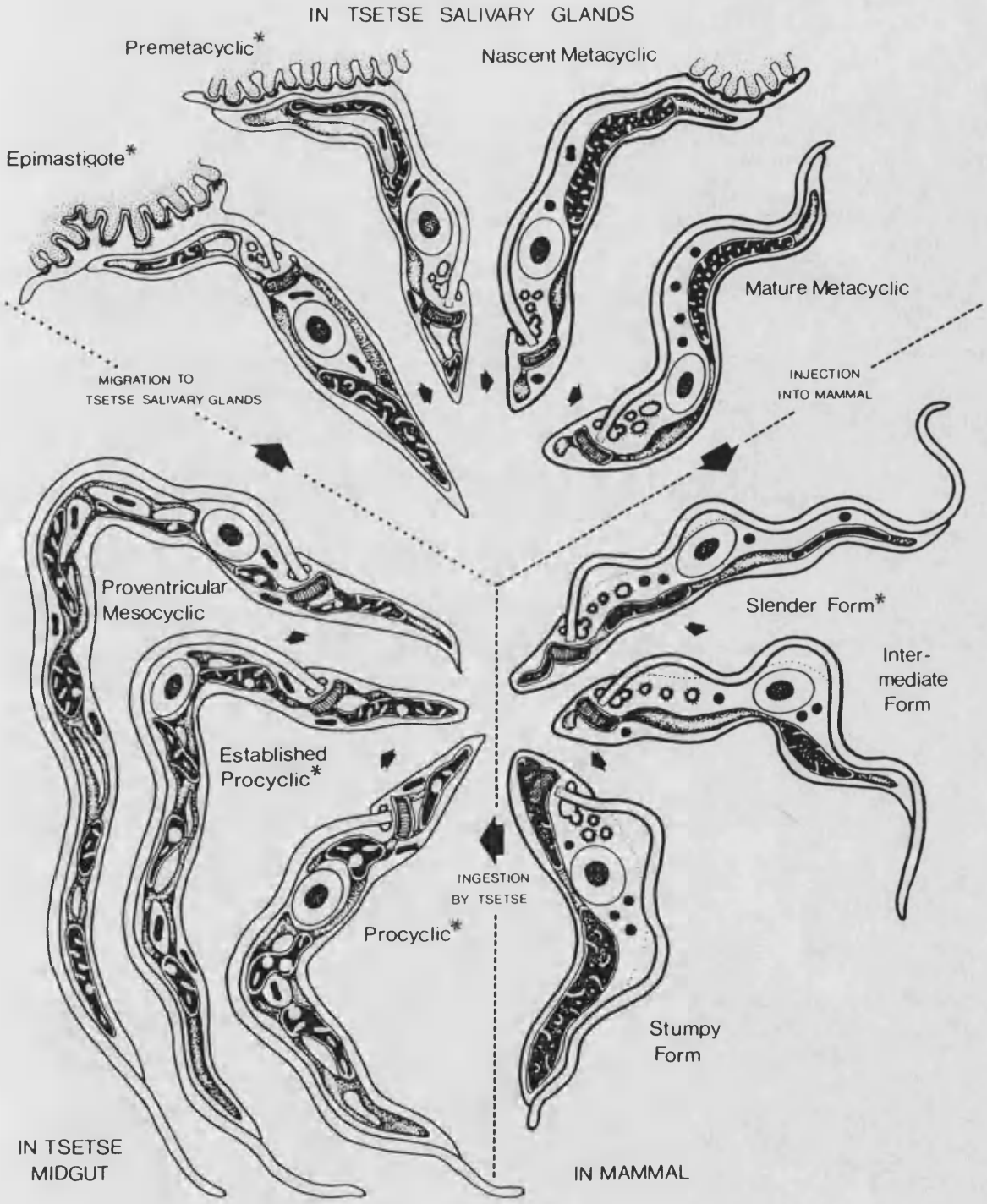

A
B

Figure 10: The developmental cycle of Trypanosoma brucei in the mammalian host and the tsetse vector showing the relative sizes of the various stages. The forms marked with an asterisk are those in which division occurs (Vickerman, 1985).


IN TSETSE SALIVARY GLANDS
Premetacyclic*
Nascent Metacyclic
Epimastigote*
Mature Metacyclic
MIGRATION TO
TSETSE SALIVARY GLANDS
INJECTION
INTO MAMMAL
Proventricular
Mesocyclic
Slender Form*
Inter-
mediate
Form
Established
Procyclic*
INGESTION
BY TSETSE
Procyclic*
Stumpy
Form
IN TSETSE
MIDGUT
IN MAMMAL

fission (Cox, 1982); recent workers however tentatively suggest the possibility of a sexual cycle in members of the brucei subgroup (Tait, 1983; Jenni et al., 1986; Vickerman, 1986).

The changes in morphology seen during the life-cycle of the trypanosome are associated with the ability of the organism to adapt its respiratory pathway to the prevailing conditions. Since the discovery that trypanosomes at certain stages of the life-cycle do not contain detectable cytochrome pigments and that their respiration is insensitive to cyanide (Ryley, 1956), it has been apparent that at least two types of respiratory pathway are present in the members of the brucei subgroup. The principle energy source of the insect stage is proline, the amino acid most abundant in the haemocoel of the tsetse fly (Srivastava and Bowman, 1971) and the transformation from mammalian to insect form is accompanied by a marked increase in proline oxidase activity (Brown et al., 1973). Proline is present at concentrations of up to 150mM, being the source of the considerable energy required by the insect for flight (Bursell, 1966). The transition from mammalian long slender to insect procyclic form, via the short stumpy form, is accompanied by the synthesis of certain enzymes of the tricarboxylic acid (TCA) cycle; isocitrate dehydrogenase, malate dehydrogenase (Kilgour, 1980), and α-oxoglutarate oxidase (Flynn and Bowman, 1973) for example. However the low levels of citrate synthase and succinate dehydrogenase seen in short stumpy forms have led Flynn and Bowman (1973) to the conclusion that the TCA cycle has minimal activity, if any, in vivo. The major end-products of the aerobic respiration of procyclic forms of the T. brucei subspecies have been determined as carbon dioxide, glutamate,

aspartate and alanine for T. b. rhodesiense, succinate, malate, fumarate and alanine for T. b. brucei and succinate and acetate for T. b. gambiense (Bowman and Flynn, 1976).

The insect forms of trypanosomes of the brucei subgroup appear to have at least three terminal respiratory systems. Of these, one has cyanide-sensitive cytochrome aa3 as its terminal oxidase, one has salicylhydroxamic-acid-sensitive α-glycerophosphate oxidase and the third, sensitive to neither cyanide nor SHAM, is as yet unknown but its identity has been suggested as cytochrome O by Njogu et al. (1980). The former two are the most important, contributing 60% and 30% respectively to the total cellular respiration (Njogu et al., 1980), these proportions possibly shifting according to the oxygen tension of the immediate surroundings of the trypanosome. Thus α-glycerophosphate oxidase has a low affinity for oxygen and cytochrome aa3 a high affinity, reflecting the high oxygen content of the mammalian bloodstream and the low oxygen tension in the tsetse midgut respectively (Hill, 1976b). Consequently it has been suggested by Njogu et al. (1980) that the main, cytochrome-mediated electron transport chain branches from the α-glycerophosphate shuttle system such that should one become inhibited the other may take over its function.

1.7: Energy production in Trypanosoma brucei

The trypanosomes which occupy the mammalian bloodstream have a markedly different physiology from those which inhabit the insect midgut. The long, slender bloodstream-dwelling trypanosome, with

which this work is solely concerned, possesses a simple mitochondrion, an incomplete TCA cycle and no functional respiratory chain (Fairlamb, 1982). Bloodstream trypomastigotes contain little if any storage carbohydrate (Opperdoes et al., 1976b) and are therefore reliant upon glycolysis for the production of energy. The human bloodstream contains glucose at approximately 1mg/ml (5mM) (Whitfield, 1979) and this constant plentiful supply of substrate eliminates the need for energy reserves in the parasite. Trypanosomal respiratory activity is correspondingly high, oxygen consumption being between 41 and 82 nmol/min/1 x 10^8 cells with glucose as the substrate, approximately fifty times the rate of respiration of mammalian cells (Bowman and Flynn, 1976).

It may be to maintain this high rate of glycolysis that in the trypanosome the major part of the glycolytic pathway is contained, with other enzymes, in a microbody-like organelle designated the glycosome by Opperdoes and Borst (1977) (Figure 7). The other enzymes sequestrated in the glycosome include adenylate kinase and enzymes of the pyrimidine biosynthetic pathway (Opperdoes and Cottem, 1982). A single trypanosome contains an average of 230 glycosomes, representing approximately 4.3% of the total cell volume. Each glycosome has an average volume of 0.0108μm^3, is spherical or ellipsoid and is surrounded by a single membrane (Opperdoes et al., 1984). As a consequence of the incomplete and non-functional TCA cycle and lack of lactate dehydrogenase in the bloodstream form (Dixon, 1966; Bird et al., 1971), glucose is metabolised almost exclusively to pyruvate under aerobic conditions, this being excreted into the bloodstream of the host (Ryley, 1956; Newton, 1968; Flynn and Bowman, 1973). The reduced

nicotinamide adenine dinucleotide (NADH) produced during the conversion of a triose phosphate to pyruvate is reoxidised by means of the α-glycerophosphate oxidase (GPO) shuttle shown in Figure 7. Located in the mitochondrion (Opperdoes et al., 1977), this terminal respiratory system is insensitive to typical inhibitors of the mammalian respiratory chain, cyanide or azide for example, and it is now generally accepted that there are no cytochromes present in the bloodstream forms of organisms of the Trypanozoon subgenus (Ryley, 1956; Ryley, 1962; Grant and Sargent, 1961). It can be seen from Figure 7 that NADH is reoxidised during the reaction catalysed by glycosomal NAD^+-linked α-glycerophosphate dehydrogenase, namely the reduction of dihydroxyacetone phosphate (DHAP) to α-glycerophosphate (α-GP). The α-GP thus produced is then oxidised back to DHAP in a reaction catalysed by mitochondrial α-glycerophosphate oxidase (Fairlamb and Bowman, 1977a). Grant and Sargent (1961) identified an α-glycerophosphate dehydrogenase component of the α-glycerophosphate oxidase, this component being capable of transferring electrons to the terminal oxidase. Difference spectra suggest that the dehydrogenase component contains flavin adenine dinucleotide (FAD) and inhibitor studies have led to the proposition that copper is present as part of the oxidase (Fairlamb and Bowman, 1977b).

It can be seen from Figure 7 that the net production of adenosine triphosphate (ATP) during aerobic respiration is +2 molecules, sufficient to maintain cellular function (Brohn and Clarkson, 1980). Under anaerobic conditions the GPO shuttle is inoperative and, assuming a conventional glycolytic scheme, the yield of ATP must be zero, cell death swiftly following. This does not occur however, and

it has long been known that the cells survive and that glucose is metabolised to approximately equimolar quantities of pyruvate and glycerol under anaerobic conditions (Ryley, 1956; Opperdoes et al., 1976b). A similar situation arises under aerobic conditions in the presence of salicylhydroxamic acid (SHAM), a metal-chelating agent which is an inhibitor of α-glycerophosphate oxidase and produces an effect which simulates anaerobiosis (Evans and Brown, 1973; Opperdoes et al., 1976b). Thus the redox balance of the trypanosome is maintained (Bowman and Flynn, 1976). In the aerobic to anaerobic transition the intracellular concentration of almost every glycolytic intermediate decreases except that of α-glycerophosphate, which rises between fourfold (Visser and Opperdoes, 1980) and thirty-fold (Hammond and Bowman, 1980a), with possible intraglycosomal concentrations of 0.1M being reached (Opperdoes and Borst, 1977). Since bloodstream forms of T. brucei are able to survive anaerobiosis it is apparent that there must be a net synthesis of ATP under such conditions, no energy reserves being present (Opperdoes et al., 1976a).

It was proposed by Bowman and Flynn (1976) that under anaerobic conditions the formation of glycerol as an end-product was by the action of a phosphatase on α-GP; this reaction however would yield no ATP. Several hypothetical schemes for the production of glycerol have been proposed by Clarkson and Brohn (1976), but that which has the most evidence to support was put forward by Opperdoes and Borst (1977) who suggest that under conditions of anaerobiosis, glycerol and ATP are synthesised from α-GP and ADP, the reaction being catalysed by glycerol kinase. The conversion of α-GP to glycerol is thermodynamically unfavourable (Mackenzie et al., 1983) but the

reaction may proceed as a result of the high local concentration of α-GP, although the necessity for an accumulation of α-GP has recently been questioned (Hammond et al., 1985). This pathway for anaerobic metabolism offers an explanation for the presence of high glycerol kinase activity (>0.5 μmol/min/mg protein) in trypanosomes of the brucei subgroup whilst those trypanosomes which do not produce glycerol anaerobically, T. cruzi and T. lewisi for example, possess relatively low levels of glycerol kinase activity (<0.03 μmol/min/mg protein) (Hammond and Bowman, 1980). Such high levels of glycerol kinase activity would otherwise be unexpected as, although glycerol is a potential substrate for trypanosomes, the plentiful supply of the preferred substrate, glucose, in the bloodstream of the mammalian host renders the utilisation of glycerol unnecessary. Further evidence to support the action of glycerol kinase in the unfavourable direction is provided by Gruenberg et al. (1980) who have shown that the transport of glycerol in T. brucei is asymmetric, the K_m for the efflux being smaller than that for the influx of glycerol. Therefore the function of the carrier is not to promote the uptake of glycerol as a substrate but rather to facilitate the elimination of anaerobically-produced glycerol so that intracellular concentrations sufficient to inhibit the conversion of α-GP to glycerol cannot be reached.

1.8: The aims of the project

From this summary it is apparent that certain aspects of trypanosomal glucose metabolism show marked differences from those of the mammalian host and may serve as the rational basis for a target

for selective trypanocidal agents. It was as a result of such research that the combination of SHAM and glycerol was suggested as a trypanocide by Clarkson and Brohn (1976) and Fairlamb et al. (1977). On administration this combination was found to clear trypanosomes from the bloodstreams of experimental animals (Evans et al., 1977; Amole and Clarkson, 1981) but a relapse of infection has been seen within one week of treatment. This relapse may be due to effective levels of both SHAM and glycerol not being reached in certain tissues (Clarkson and Brohn, 1976; Poltera, 1985), now tentatively identified by Abolarin et al. (1982) as the choroid plexus of the brain. The combination of SHAM and glycerol, although theoretically well-founded, does not appear to be of practical use as a treatment of human or animal trypanosomiases however, due to the large, almost lethally-toxic dosage which would be necessary (van der Meer and Zwart, 1980; Meshnik, 1984).

Continued elucidation of the metabolic pathways of trypanosomes may reveal further reactions which may, either singly or in combination, be exploited and it is to this end that the work presented here has been carried out. The initial aim of the project was to investigate the effect of varying oxygen concentration in the range between the two extremes of glucose metabolism, the aerobic and anaerobic glycolytic pathways. As has previously been stated the main product of glycolysis in trypanosomes under aerobic conditions is pyruvate while under anaerobic conditions pyruvate and glycerol are produced in approximately equimolar quantities; aerobically the net reaction occurring is:

$$\text{Glucose} + O_2 \longrightarrow 2\text{pyruvate} + 2H_2O$$

whilst anaerobically,

$$\text{Glucose} \longrightarrow \text{pyruvate} + \text{glycerol}.$$

Determination of the point at which aerobic metabolism becomes inoperative and anaerobic metabolism takes over as the primary energy source may identify an enzyme or enzymes which play a key role in the control of the "switchover". Further investigation of the relevant enzymes may indicate any susceptibility to action by inhibitors which could be developed as drugs.

In the second part of this project an attempt was made to purify trypanosomal NAD^+-linked α-glycerophosphate dehydrogenase (α-GPDH) (EC 1.1.1.8.) with a view to preliminary kinetic studies on the enzyme. The NAD^+-linked α-GPDH of T. brucei, located in the glycosome (Figure 11), is particularly interesting for two reasons. Firstly it plays an important role in trypanosomal metabolism under both aerobic and anaerobic conditions, catalysing the reaction which brings about the reoxidation of NADH ie:

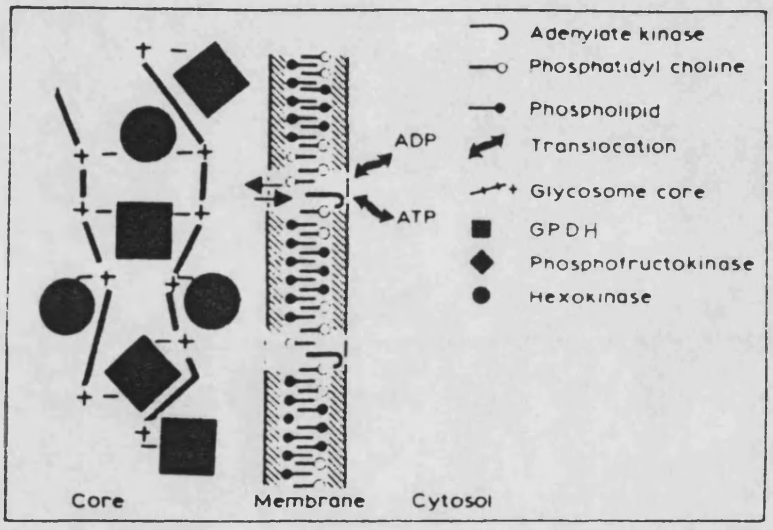
$$\text{DHAP} + \text{NADH} \longrightarrow \alpha\text{-GP} + NAD^+$$

Secondly, it is the only enzyme of which the product concentration seems to rise under anaerobic conditions. Therefore the aim of the work presented here was to quantify, using a pure enzyme preparation, the kinetic parameters of α-GPDH from T. brucei brucei and in

particular the effect on enzyme activity of the high levels of α-GP reported to occur under anaerobic conditions.

It is apparent that the work carried out falls into various separate subject categories and it will therefore be presented as such. Chapter 2 refers to general methods used in the preparation of trypanosomes, Chapter 3 to the investigation of the aerobic/anaerobic transition of glucose metabolism and Chapters 4 and 5 relate to the purification of trypanosomal α-GPDH and investigation of its kinetic behaviour respectively. A discussion of this work is presented in Chapter 6.

Figure 11: The proposed disposition of α-GPDH within the glycosome of T. rhodesiense, shown bound to the core of the glycosome by weak ionic interactions (McLaughlin, 1985).


Adenylate kinase
Phosphatidyl choline
Phospholipid
Translocation
Glycosome core
GPDH
Phosphofructokinase
Hexokinase
ADP
ATP
Core
Membrane
Cytosol

CHAPTER 2: GENERAL MATERIALS AND METHODS

Unless otherwise stated all procedures were carried out at 4°C.

2.1: Materials

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co., Poole, Dorset and were reagent grade. The ion-exchangers DEAE-cellulose and CM-cellulose were purchased from Whatman Ltd., Maidstone, Kent. Roswell Park Memorial Institute medium Formulation 1640 (RPMI medium 1640) was obtained from Flow Laboratories, Irvine, Scotland.

Ringer-phosphate buffer was prepared essentially as described by Ryley (1955) thus:

KH_2PO_4	22mM
NaCl	98mM
$MgSO_4$	1mM
KCl	2mM
Final pH	8.0

Ringer-phosphate(G) buffer was Ringer-phosphate buffer containing glucose at a concentration of 10mM while Ringer-phosphate(SG) buffer contained 10mM glucose and sucrose at 3g/100ml.

2.2: Organisms used

Stocks of clones of the long, slender bloodstream form of

Trypanosoma brucei brucei strain EATRO 427-12/ICI-060 were kindly supplied by Dr. H. P. Voorheis and stored in sealed capillary tubes in liquid nitrogen. When required, a vial of these clones was removed from the liquid nitrogen and 1 x 10^7 viable trypanosomes used to infect a single rat (see below). At 71 hours post-infection the rat was anaesthetised with diethyl ether and bled by aortic section into a syringe containing heparin and this blood was mixed with glycerol to a final concentration of 13% (w/v). Small volumes (approximately 25μl) were then drawn up into glass capillary tubes and these tubes, sealed at both ends, stored in liquid nitrogen. These so-called "stabilates" (Lumsden and Hardy, 1965) were then used to provide viable trypanosomes for infection into rats (Lumsden, 1972) when cells were required for experimentation.

2.3: Estimation of cell numbers

The number of trypanosomes present in a sample was estimated by microscopic examination of a suitably diluted sample using a Gallenkamp Neubauer haemocytometer-type counting chamber.

2.4: Infection of the host

Cells for injection into the host were obtained by dilution of the contents of several vials of stabilate each with 2ml Ringer-phosphate(G) buffer. The trypanosomes were counted and a volume containing 1 x 10^7 cells was injected intraperitoneally into a rat approximately 200g in weight. If the volume containing the

required number of cells was greater than 1ml, a further vial of stabilate was added to the existing cell suspension and the total volume adjusted until the cells were contained in a volume sufficiently small for an injection causing no discomfort to the host. The harvesting of these cells 71 hours post-infection (see below) yielded an average 12ml blood per rat at 5 x 10^8 cells/ml.

2.5: Isolation of trypanosomes from the host

The method described here was adapted from that of Lanham and Godfrey (1970). At approximately 71 hours post-infection the host rat was anaesthetised with diethyl ether and blood was withdrawn from the bifurcation of the common iliac artery with the aorta, situated in the abdominal cavity. The blood was collected into a syringe of 20ml volume into which had previously been placed 1ml heparin solution (200U/ml Ringer-phosphate buffer). The blood was spun in a BTL bench centrifuge at 800 x g for ten minutes.

Trypanosomes spun down as a white "buffy coat" above the packed cells but below the plasma. The plasma was removed by aspiration and replaced with Ringer-phosphate(SG) buffer to 2/3 of the plasma volume. The buffy coat was suspended in this volume by gentle agitation and was then transferred to a clean tube and centrifuged as before. This washing procedure was repeated several times before resuspended cells were placed onto a Whatman DEAE-cellulose anion-exchange column. This column had been equilibrated with Ringer-phosphate(SG) buffer and trypanosomes were eluted with the same buffer.

This method separated trypanosomes from blood components by

virtue of differing surface-charge, the more negatively-charged blood cells and platelets being retained while trypanosomes were eluted and collected ready for experimental work.

CHAPTER 3: INVESTIGATION OF THE AEROBIC/ANAEROBIC TRANSITION OF GLUCOSE METABOLISM IN TRYPANOSOMA BRUCEI

3.1: Methods used

3.1.1: The incubation of trypanosomes under varying oxygen tensions

The metabolism of trypanosomes under conditions intermediate to the two extremes, total aerobiosis and total anaerobiosis, was investigated by the incubation of trypanosomes under conditions of varying oxygen tension and the subsequent assay, over a time course, of the products of metabolism.

Initial investigations were carried out using a Rank-type Clark electrode (Figure 12a) with an attached chart recorder. A 2ml aliquot of Roswell Park Memorial Institute medium, Formulation 1640 (RPMI medium 1640), pH 7.4 and containing 10% (w/v) horse serum, was equilibrated to 37°C in the electrode reaction vessel with the relevant mixture of oxygen and nitrogen being introduced into the medium *via* a syringe needle "cannula" prior to commencement of the experiment. A pellet containing sufficient trypanosomes to give a final concentration of 1×10^8 cells/ml was resuspended in 0.5ml RPMI medium 1640 and introduced into the reaction vessel by removal of the rubber bung, this operation being performed as quickly as possible.

As incubations were performed using this apparatus, several problems were experienced and adjustments made to the system in an effort to overcome them:

1) The initial pH of the RPMI medium 1640 was adjusted to 8.1 in order that any fall in pH due to the production of pyruvate during the period of incubation adversely affected neither cell numbers nor the observed rate of glycolysis (Brohn and Clarkson, 1980). That this was so was determined experimentally (see below).

2) The presence of 10% (w/v) horse serum in the incubation medium led to frothing and the consequent seepage of medium from the reaction vessel *via* the gas-escape cannula. The substitution of bovine serum albumin (BSA) at a concentration of 150mg/100ml (150mg%) led to a reduction in the extent of foaming which occurred.

3) Frothing was further reduced by the introduction of the gas mixture *via* the cannula into the space above the incubation medium rather than directly into the medium itself.

4) The 0.5ml volume of RPMI medium 1640 in which the trypanosomes were introduced into the reaction vessel was itself pre-equilibrated to 37^{o}C and the required oxygen tension. The deviation from that oxygen tension, as shown by the trace on the associated chart recorder, was 1/10 of that seen when no pre-gassing was carried out and the time taken for the incubation mixture to return to the required oxygen tension was similarly only 1/10 of that previously experienced.

5) In order that the oxygen tension of the medium should be easier to maintain, the trypanosome density was reduced to a final

concentration of 5×10^7/ml.

Although the above refinements led to more reliable results being obtained other problems proved less simple to solve. The considerable depletion of the incubation volume upon the removal of up to four samples, each of sufficient volume for cell counts and pyruvate and glycerol assays to be performed upon them, finally led to the discontinuation of use of the Clark electrode and the design and manufacture of a more suitable piece of equipment (Figure 12b).

The multi-necked glass flask produced incorporated a probe-type oxygen electrode (Model 507, LH Fermentation Ltd., Stoke Poges, Bucks.) with an associated meter. This electrode was more sensitive to changes in oxygen tension and was also more quick to react to these fluctuations than the Clark electrode. Published values for the *in vivo* oxygen tensions of the human blood (Cantarow and Trumper, 1962) suggested that the investigation of metabolism over a range of relatively low oxygen concentrations would be realistic and to this end a mixture of nitrogen and air was substituted for an oxygen/nitrogen mixture, the former meaning that low values for oxygen tension were more easily achieved. The incoming mixtures of nitrogen and air in the correct proportions were humidified prior to entering the reaction vessel by being bubbled through two 100ml volumes of water, both maintained at 37^{o}C.

The volume of the incubation mixture was raised to 75ml at a density of 2×10^7 cells/ml. The incubation medium was again RPMI medium 1640 at pH 8.1. Protein was incorporated as BSA at 150mg%, this concentration being found to be sufficient to prevent cell lysis

and to maintain the rate of glycolysis. To each 75ml volume was added 30µl anti-foam (BDH Chemicals, Poole, England) diluted 1/10 with distilled water prior to use (Brohn and Clarkson, 1978). The flask was held in a water-bath at 37°C throughout each experiment and the RPMI medium 1640 was pre-incubated to this temperature and equilibrated with the required gas mixture before the addition of trypanosomes. The trypanosomes, which had been eluted from a DEAE-cellulose column with Ringer-phosphate(SG) buffer, were centrifuged at 800 x g for ten minutes at 4°C and the resulting pellet resuspended in 1ml of the pre-equilibrated RPMI medium 1640 which was withdrawn from the flask via the large-bore syringe needle. This suspension, introduced slowly into the incubation medium by the same route, caused minimal fluctuation in the oxygen tension of the medium. At each of several pre-determined times a 1ml sample was withdrawn via the syringe needle. A small volume was retained for estimation of cell numbers and the remainder centrifuged briefly in a Sarstedt microfuge. The cell-free supernatant was frozen until assay.

Subsequent assay showed that even under aerobic conditions, with 100% air being bubbled through the medium, some glycerol was always present in these samples and so the trypanosome density was further reduced to 1×10^7 cells/ml to minimise the possibility that oxygen utilisation was faster than the rate of supply or that an anaerobic microenvironment was being created around many or all of the trypanosomes due to "overcrowding".

Figure 12a: The Rank-type Clarkoxygen electrode

A,B - circulation in water jacket

C - incubation medium

D - electodes

E - magnetic 'flea'

F - gas escape cannula

G - gas inlet

Figure 12b: The multi-necked flask designed for the incubation of trypanosomes under varying oxygen tensions. The body of the flask was immersed in a 37^{o}C water-bath throughout

A - probe-type oxygen electrode

B - magnetic stirring apparatus

C - glass sinter

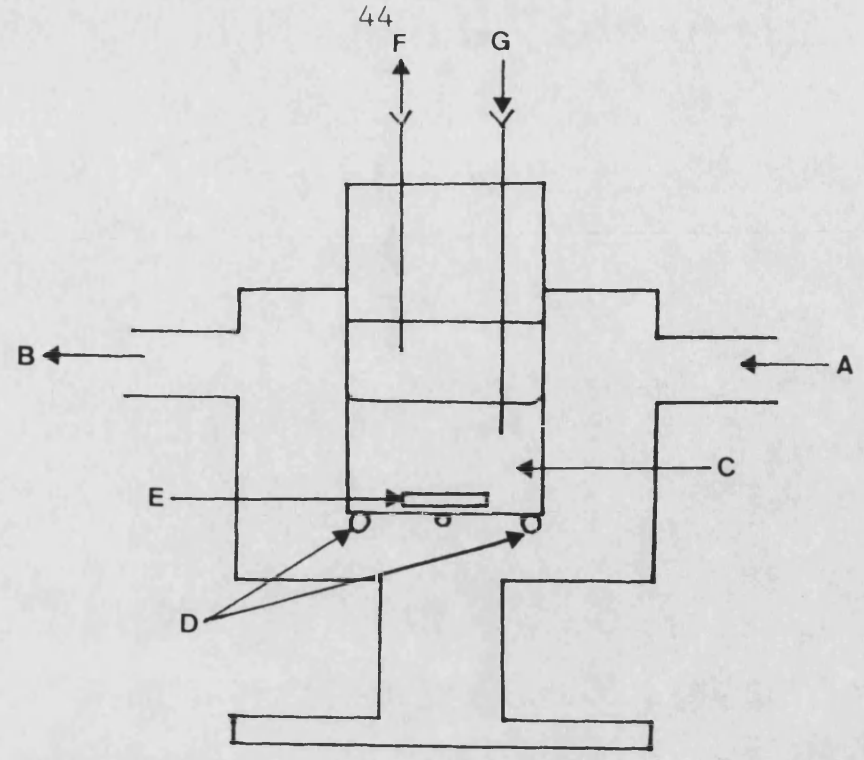
D - incubation mixture

E - large-bore syringe needle for the introduction of trypanosomes and withdrawal of samples

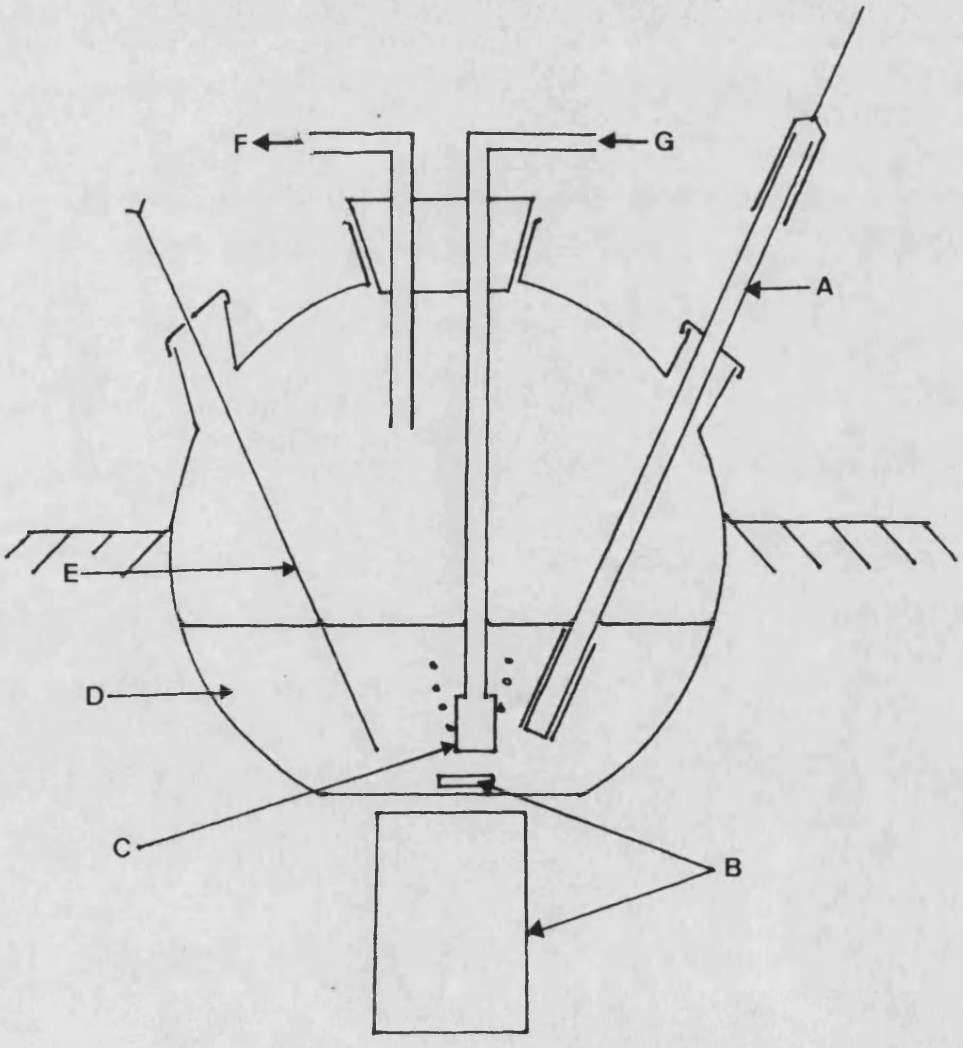
F - gas outlet

G - gas inlet

A



B



3.1.2: Investigation of the maintenance of metabolic integrity during incubation

The following procedure was used to determine whether the metabolic integrity of the cells was maintained throughout a period of incubation. At suitable time-points during an incubation, carried out in the multi-necked flask shown in Figure 12b, 5ml aliquots were removed *via* the syringe needle and spun in a BTL bench centrifuge at 800 x *g* for ten minutes. The pellet of trypanosomes was resuspended in 1ml RPMI medium 1640 and transferred to an eppendorf tube. This suspension was spun briefly in a Sarstedt microfuge and the pellet resuspended in 0.2ml RPMI medium 1640. The resulting cell suspension was added to 1.27ml Ringer-phosphate buffer in the reaction vessel of a pre-calibrated Rank-type Clark Oxygen electrode (Figure 12a). A base-line was quickly established on the attached chart recorder and then 30μl 500mM glucose solution was added *via* a syringe needle in the airtight bung to yield a final glucose concentration of 10mM in a total volume of 1.5ml. The rate of oxygen consumption was then calculated from the traces obtained from the chart recorder. The number of cells in each sample was determined and the rate of oxygen consumption by 1 x 10^8 trypanosomes calculated.

3.1.3: The assay of pyruvate and glycerol

The quantitative estimation of pyruvate and glycerol produced during glycolysis was most conveniently performed within a single

system, the reactions occurring being:

A) Glycerol + ATP $\xrightarrow{GK}$ α-glycerophosphate + ADP

B) ADP + phosphoenolpyruvate $\xrightarrow{PK}$ ATP + pyruvate

C) Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ lactate + NAD^+

where GK is glycerol kinase, PK is pyruvate kinase and LDH is lactate dehydrogenase.

The reaction was followed spectrophotometrically at 340nm and 25°C, estimating the extent of oxidation of NADH to NAD^+, using a digital PYE Unicam SP8-100 UV spectrophotometer. The assay system, adapted from Bergmeyer (1974) was as shown in Table 1.

Assay components 1 to 3 were placed into a 1ml plastic cuvette, the absorbance noted and the sample added to start the reaction. The reaction shown as reaction C in the above scheme was then taking place and the concentration of pyruvate in the sample being assayed. The point at which no further change in absorbance was apparent, indication that the reaction was complete, was always within the ten minutes given. The addition of an aliquot of the glycerol kinase/pyruvate kinase solution to the same cuvette initiated reaction

A and consequently reactions B and C, and thus the quantity of glycerol in the sample was estimated over a 15 minute period. Although the reaction sequence had not reached completion within this time, preparation of and reference to standard curves indicated that the extent to which the reaction had run was proportional to the concentration of glycerol in the sample and the period of time allowed for the reaction to run was therefore satisfactory. Standard solutions were prepared in RPMI medium 1640 using stock 10mM solutions of pyruvate and glycerol to yield final concentrations of each of between 0 and 1.5mM. It was ascertained that the assay of neither glycerol nor pyruvate was affected by the relative concentration in the sample of the complementary substrate. Standard curves were constructed for each set of assays thus taking into account changes in the temperature of the environment or slight inaccuracies in the preparation of assay solutions. Both standards and samples were assayed in duplicate. Typical standard curves are shown in Figure 13.

3.1.4: The assay of glucose

The incubation medium used, RPMI medium 1640, contained glucose at a concentration of 11mM (2g/litre) and it has been estimated that T. brucei may consume glucose at a rate of approximately 4.8 μmol/1 x 10^8 cells/hour (Brohn and Clarkson, 1980). It is therefore apparent that the assay system for the estimation of glucose was required to be sufficiently sensitive and accurate to detect the small changes in glucose concentration likely to occur.

The simple and widely-used colorimetric assay for the estimation

of glucose which involves the use of glucose oxidase, peroxidase and o-dianisidine as an indicator (Keston, 1956) unfortunately proved to be inaccurate and unreliable. The assay system eventually used was that involving the following reactions:

A) $\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ATP}$

B) $\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADH} + \text{H}^+$

where HK is hexokinase and G6PDH is glucose-6-phosphate dehydrogenase. The system used was as shown in Table 2, the reaction being carried out at 25°C in a 1ml plastic cuvette and followed spectrophotometrically at 340nm.

All reagents were equilibrated at room temperature except the enzyme solution which was kept on ice. A standard curve was constructed on each occasion that samples were assayed, the standards being prepared in distilled water over the range 9.0 to 11.5mM glucose. It was established that the slight pink coloration of the sample did not affect the absorbance of the assay at 340nm.

Samples and standards were all assayed in triplicate which, combined with the use of a Cecil CE588 High Performance Microcomputer Scanning spectrophotometer which read absorbance values to three decimal places, led to this method being both accurate and reproducible. A typical standard curve is shown as Figure 14.

Table 1: Method for the estimation of pyruvate and glycerol

Reagent	Initial concentration	Volume
1) Buffer, pH 7.6		
Triethanolamine	0.4 M	
EDTA	2.0mM	790µl
Mg^{2+}	4.0mM	
K^{+}	4.0mM	
2) NADH	4.0mM	
ATP	5.0mM	100µl
Phosphoenolpyruvate	5.0mM	
3) LDH	5U per assay	10µl
4) Sample		10µl
After ten minutes the change in absorbance ($A_{t=0} - A_{t=10}$) was noted		
5) GK	5U per assay	
PK	5U per assay	5µl
After a further 15 minutes the change in absorbance ($A_{t=10} - A_{t=25}$) was noted		

Table 2: A method for the estimation of glucose

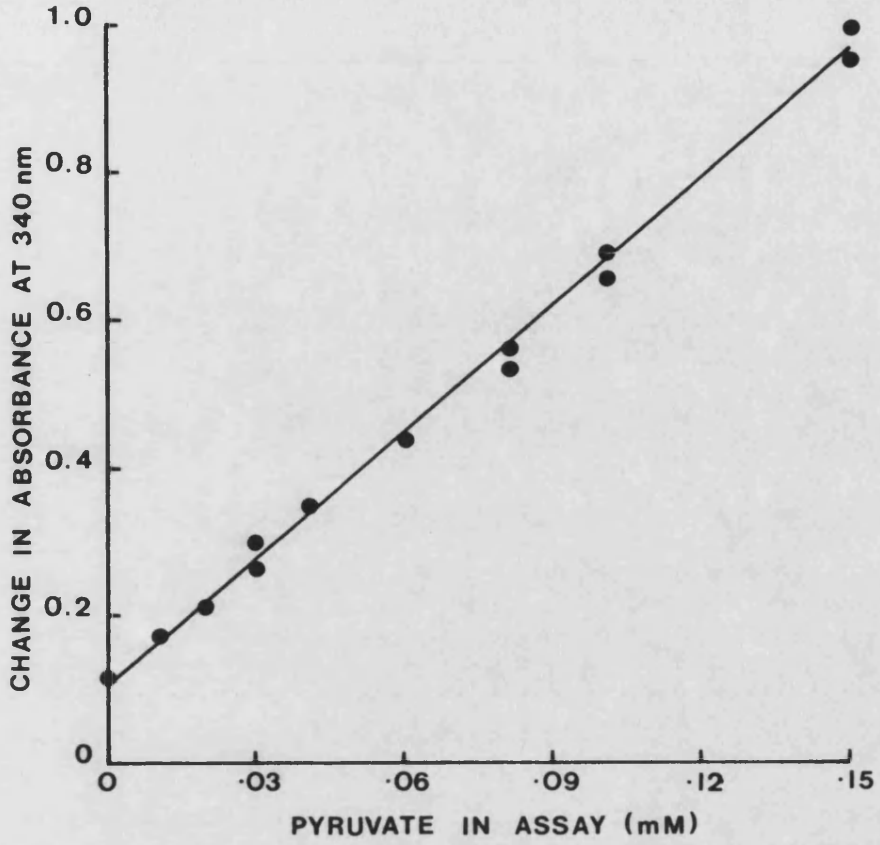
Reagent	Initial Concentration	Volume (μl)
Buffer, pH 7.5:		
Triethanolamine	0.3 M	
EDTA	2.0mM	600
$Mg2^+$	4.0mM	
NAD^+	10.0mM	
ATP	50.0mM	200
Sample	Diluted 1/5 with buffer	100
An initial absorbance (A_o) value was obtained against air		
HK	1U per assay	
G6PDH	1U per assay	100
After 25 minutes the absorbance versus air was again noted (A_{25}) and the change in absorbance ($A_o - A_{25}$) used to estimate the concentration of glucose from a standard curve.		

Figure 13: Typical standard curves used in the estimation of

a) pyruvate and

b) glycerol

A single system was used to estimate pyruvate and glycerol, the reactions occurring (see page 46) being followed spectrophotometrically at 340nm and 25°C. The assay system is described in Table 1 on page 49.


1.0
0.8
0.6
0.4
0.2
0
CHANGE IN ABSORBANCE AT 340 nm
0
·03
·06
·09
·12
·15
PYRUVATE IN ASSAY (mM)

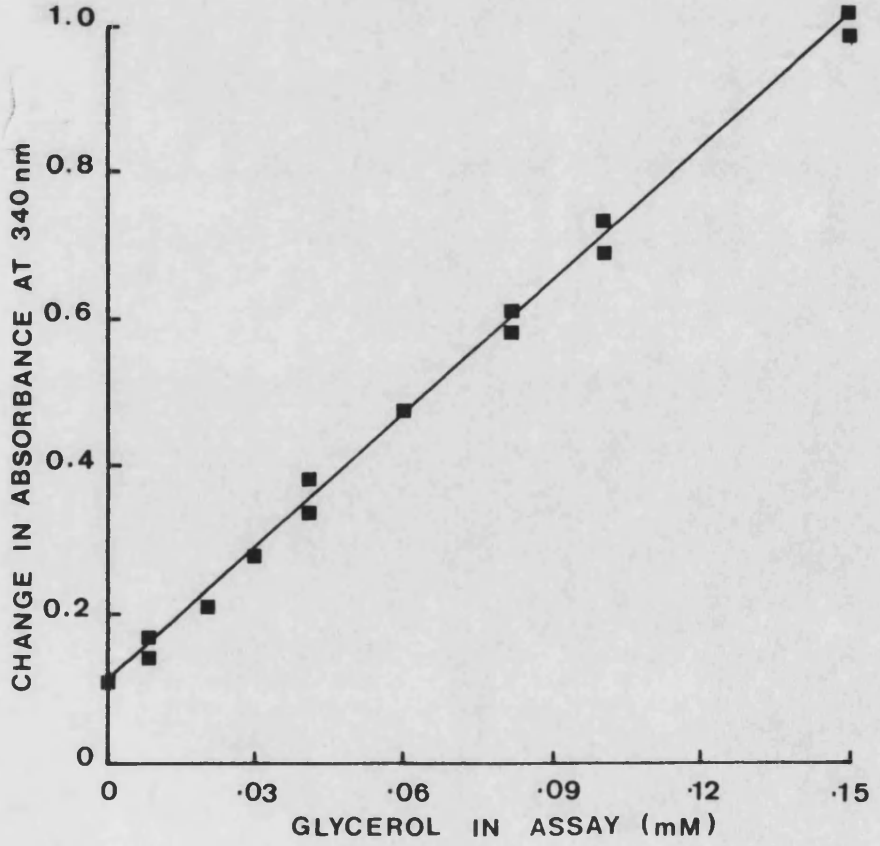
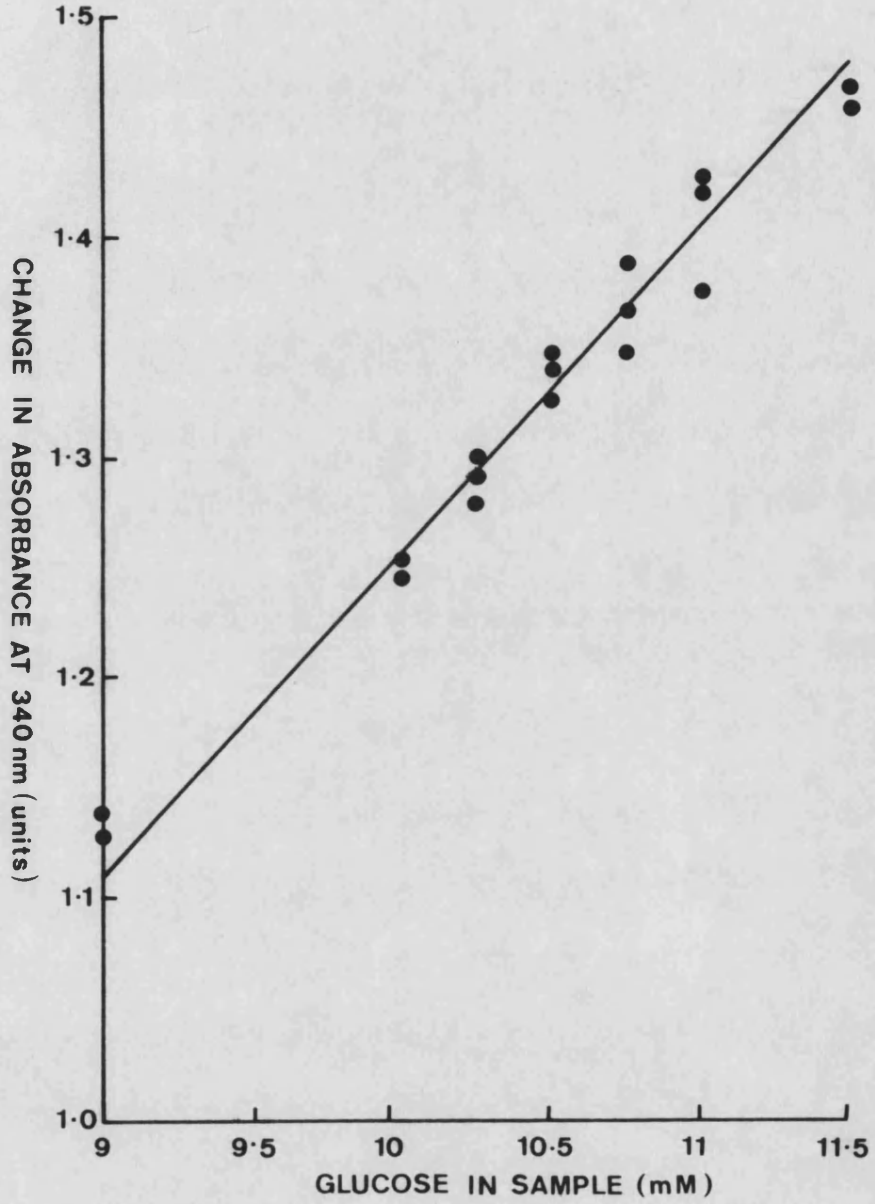

1.0
0.8
0.6
0.4
0.2
0
CHANGE IN ABSORBANCE AT 340 nm
0
·03
·06
·09
·12
·15
GLYCEROL IN ASSAY (mM)

Figure 14: A typical standard curve used in the estimation of glucose. The assay system used is shown in Table 2 on page 50, the reaction being followed at 340nm and 25°C.


1·5
1·4
1·3
1·2
1·1
1·0
CHANGE IN ABSORBANCE AT 340nm (units)
9
9·5
10
10·5
11
11·5
GLUCOSE IN SAMPLE (mM)

3.2: Results obtained

3.2.1: The assay of metabolites

The assay of pyruvate, glycerol and glucose for each set of samples was accompanied by the production of a standard curve obtained with the same enzyme and co-enzyme solutions used in the assay of experimental samples. A computer program was devised which calculated the various parameters for each curve and also, from experimental values, the concentration of substrate present and a standard error for that concentration ie the range of possible concentrations in a sample. In the calculation of the glycerol:pyruvate ratios shown in Figure 15, the standard errors were compounded using formulae widely-quoted, by Faires and Parkes (1964) for example.

3.2.2: The maintenance of metabolic integrity

Rates of oxygen consumption were obtained at several time-points during incubations at various oxygen tensions. All showed that, under the conditions used, the rate of oxygen consumption did not decrease significantly during the period of incubation. Table 3 shows a typical set of results.

Table 3: The rate of oxygen consumption by trypanosomes incubated at 0.6% oxygen

Time after start of incubation (minutes)	Rate of oxygen consumption (nmol/l x 10^8 cells/min)
1	44.6
30	42.8
60	37.1

3.2.3: Investigation of the transition between aerobic and anaerobic glycolysis

Samples of medium from incubations at various oxygen tensions were taken at several times during the experiment and assayed for the presence of pyruvate and glycerol and the disappearance of glucose. The results obtained are shown in Figure 15, plotted as the ratio of glycerol:pyruvate produced after 60 minutes incubation. The choice of 60 minutes as the period of incubation at which the ratio was calculated was arbitrary, the ratio having been determined as independent of sampling-time between 30 and 75 minutes. Figures 16a, b and c show composite results obtained from incubations at three different oxygen tensions. The rate of production of glycerol plus pyruvate was very similar at each oxygen tension and under anaerobic conditions glycerol and pyruvate were produced at approximately equal

rates. At higher oxygen tensions, pyruvate became the major product but glycerol was always produced in small but significant amounts. It has been reported by Opperdoes et al. (1976) that glucose is utilised at the same rate under aerobic and anaerobic conditions and, although not represented in Figure 16, within the limits of experimental error the sum of the amounts of pyruvate and glycerol consumed accounted for the quantity of glucose consumed at every oxygen tension.

The curve in Figure 15 was fitted to the model described below. The following assumptions were made:

1) That the rate of utilisation of glucose was constant at all oxygen tensions, this having been proved experimentally to be so, and consequently,

2) that the total concentration of pyruvate and glycerol produced was also constant at all oxygen tensions.

Aerobic glycolysis was represented thus:

$$\text{Glucose} + O_2 \longrightarrow 2\text{pyruvate} + 2H_2O \quad \text{(reaction 1)}$$

and anaerobically,

$$\text{Glucose} \longrightarrow \text{pyruvate} + \text{glycerol} \quad \text{(reaction 2)}$$

Then V = the maximum rate of pyruvate production via the aerobic pathway (reaction 1) at full oxygen saturation, and also the rate of

glycerol plus pyruvate production at all levels of aerobiosis,

and v = the rate of pyruvate production *via* the aerobic pathway (reaction 1) at submaximal oxygen tensions.

As it was assumed that glucose was consumed at the same rate under all oxygen tensions then the rate of glycerol or pyruvate production at any level of anaerobiosis *via* the anaerobic pathway (reaction 2) was

$$\frac{(V - v)}{2}$$

Then

$$\frac{(\text{glycerol})}{(\text{glycerol}) + (\text{pyruvate})} = \frac{V - v}{2V}$$

Rearranged, this reaction becomes equation 1:

$$\frac{(\text{glycerol})}{(\text{pyruvate})} = \frac{V - v}{V + v}$$

It was assumed that the dependence of the rate of pyruvate production aerobically (*via* reaction 1) on oxygen concentration was Michaelian *ie*

$$v = \frac{V(O_2)}{K + (O_2)}$$

where K is the "K_m" for the aerobic pathway. Substitution for v in equation 1 yielded the following:

$$\frac{(\text{glycerol})}{(\text{pyruvate})} = \frac{K}{K + 2(O_2)}$$

which tends to zero when the oxygen concentration is high and becomes unity when the oxygen concentration is zero. In fact the actual equation to which the curve was fitted, by the method of least squares, was

$$\frac{(\text{glycerol})}{(\text{pyruvate})} = \frac{K}{K + 2(O_2)} + C$$

where C was a constant included to correct for the small but significant amount of glycerol found in every incubation carried out. From these results (Figure 15) the value obtained for K was 3.6 $\pm$ 0.8mmHg, corresponding to an approximate oxygen concentration of 5.0 μM.

Figure 15: The ratio of glycerol:pyruvate concentrations produced by T. brucei incubated in RPMI medium 1640 with 11mM glucose at various oxygen tensions (see page 40 for experimental details). The incubation medium was maintained at 37°C throughout and samples removed and rendered cell-free for assay. The graph opposite represents the results obtained from samples withdrawn after 60 minutes of incubation.

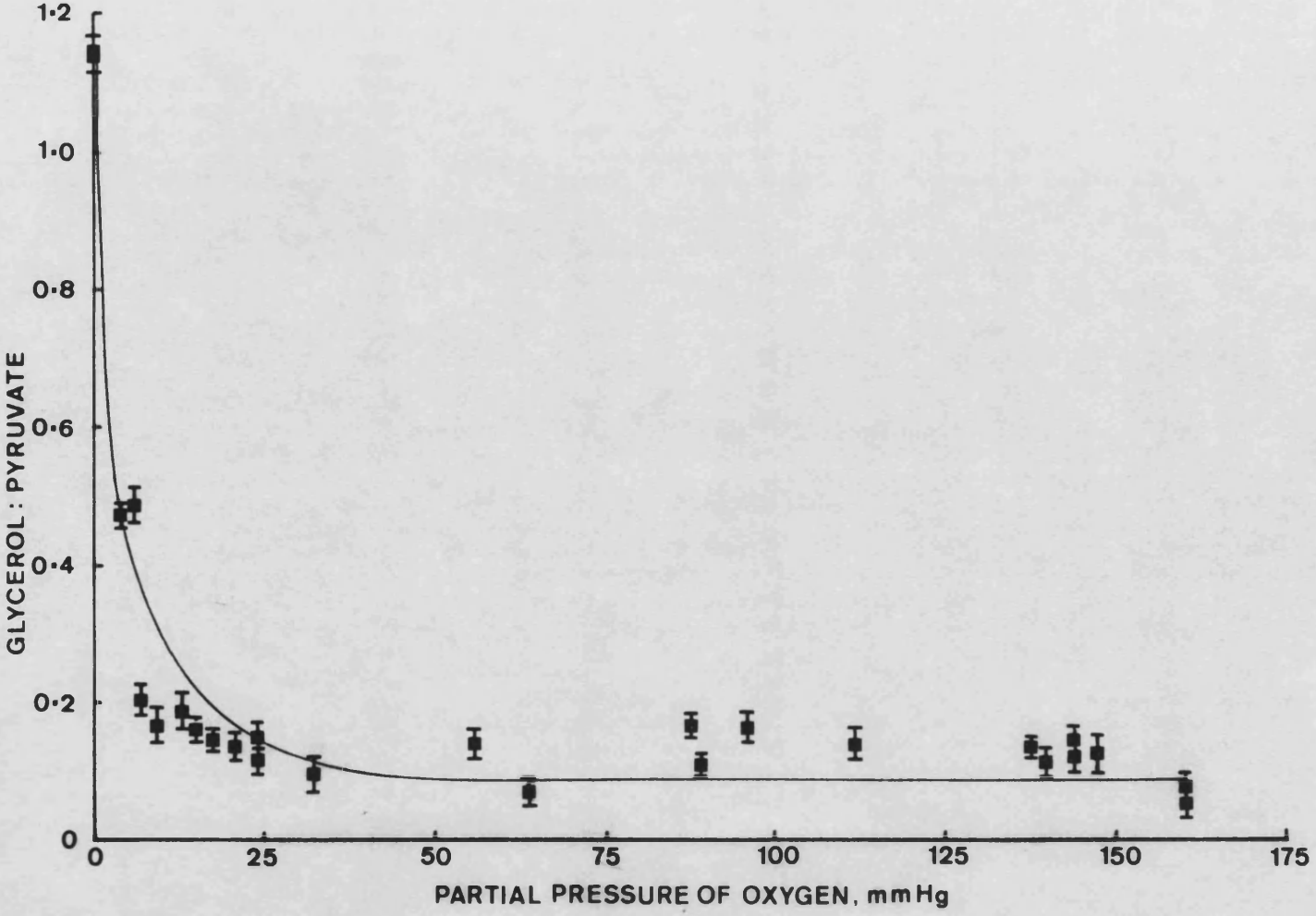

1·2
1·0
0·8
0·6
0·4
0·2
0
GLYCEROL : PYRUVATE
0
25
50
75
100
125
150
175
PARTIAL PRESSURE OF OXYGEN, mmHg

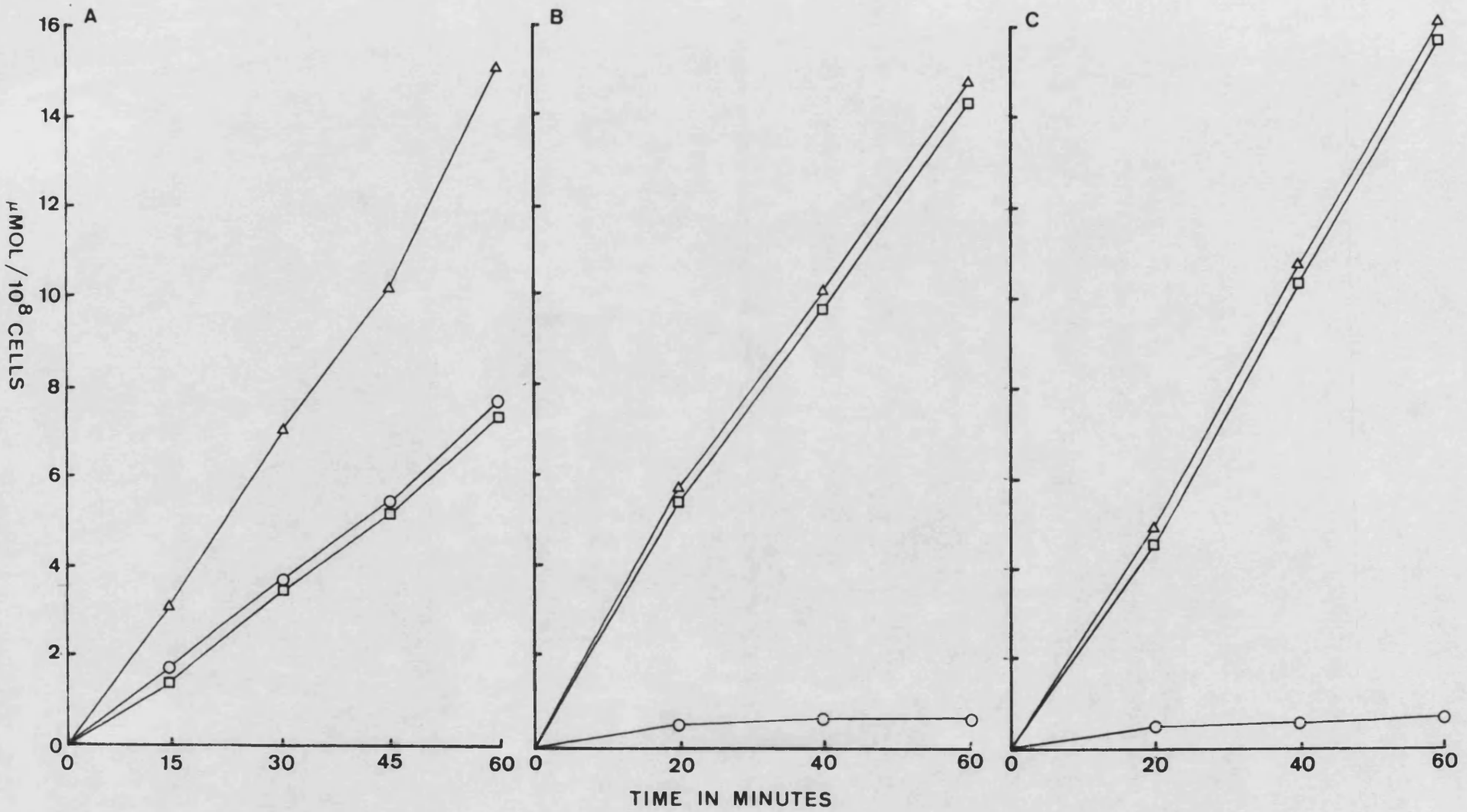
Figure 16: The production of glycerol (O) and pyruvate (□) from glucose (Δ) by T. brucei at oxygen tensions of

a) 0mmHg

b) 16mmHg

c) 160mmHg

Trypanosomes were incubated at a concentration of 10^7/ml in 75ml RPMI medium 1640, pH 8.1, at 37°C in a multi-necked flask (Figure 12b) under each of the above oxygen tensions. Protein was incorporated as BSA at 150mg%. Samples were removed after 15, 30, 45 and 60 minutes incubation and, having been rendered cell-free, assayed for concentrations of glycerol, pyruvate and glucose.


A
B
C
16
14
12
10
8
6
4
2
0
μMOL/10⁸ CELLS
0
15
30
45
60
0
20
40
60
0
20
40
60
TIME IN MINUTES

CHAPTER 4: THE PURIFICATION OF TRYPANOSOMAL α-GPDH

4.1: The routine assay of NAD^+-linked α-GPDH activity

The amount of α-GPDH activity in any extract was most conveniently measured spectrophotometrically, following the oxidation of NADH at 340nm and 25°C:

$$DHAP + \alpha\text{-NADH} + H^+ \longrightarrow \alpha\text{-GP} + NAD^+$$

Previous workers have suggested various assay systems (Grant and Sargent, 1960; Reynolds, 1975; Misset and Opperdoes, 1984) but that used, since it gave an acceptable rate and was both inexpensive and simple, was as follows:

50mM phosphate buffer containing 8mM $MgCl_2$, pH 7.4	(900-x)μl
2mM NADH	
5mM DHAP	100μl
Extract to start reaction	xμl

where the volume of extract (x) required was usually between 10 and 50 μl. The total assay volume was 1ml. Throughout this work, one unit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1μmol NAD^+ in one minute under the conditions described above.

4.2: Determination of protein concentration

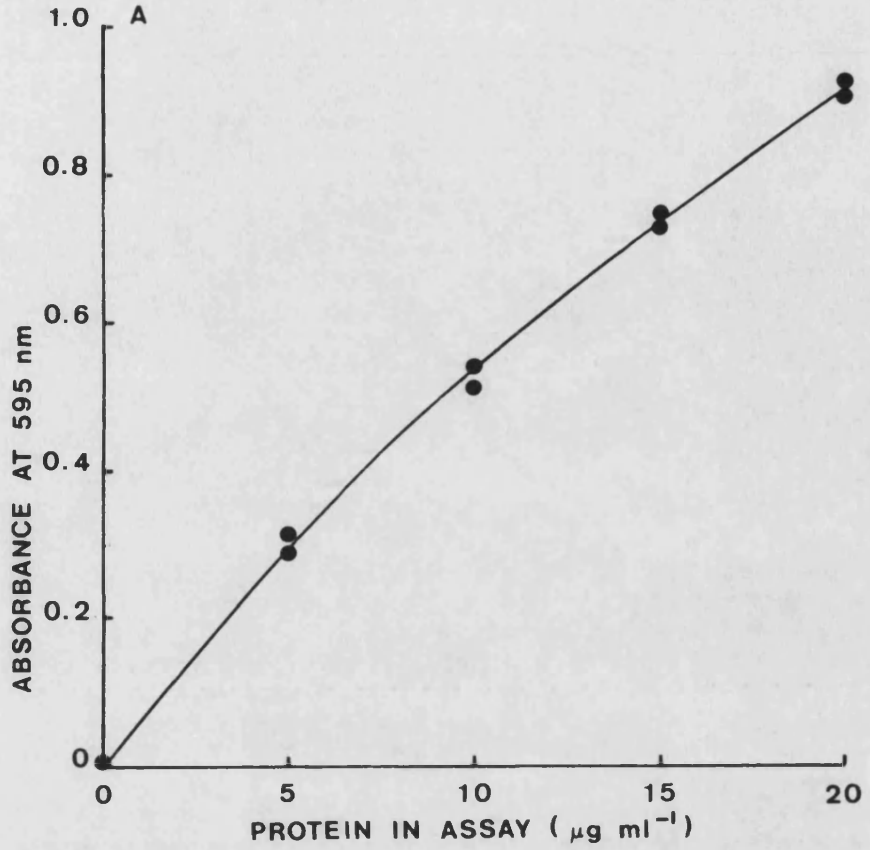
The concentration of protein in any solution was determined by one of two methods, the first being that described by Lowry *et al.* (1951). Although widely used, this method has certain disadvantages, one of which being that it is time-consuming particularly during the initial stages of an enzyme purification when a suitable sample volume may not immediately be apparent. In order to overcome this, a second method was used, based on the dye-binding method of Bradford (1976). The so-called 'Bio-Rad' method is a quick, simple one-step reaction (Bio-Rad Protein Assay Instruction Manual, 1984).

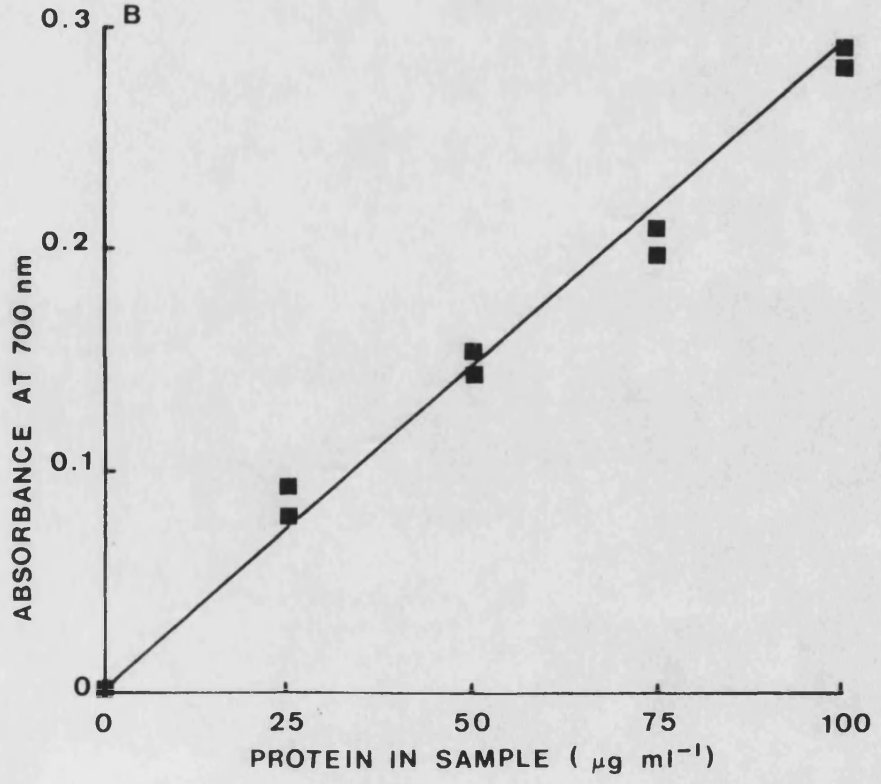
Although for both methods use was made of bovine serum albumin for preparation of a standard curve (Figure 17), a given sample yielded significantly different apparent protein concentrations when assayed by either method. It has been shown that the method of Lowry *et al.* (1951) is the more accurate (Bio-Rad Protein Assay Instruction Manual, 1984) and was therefore used during this work when absolute protein concentrations were required. When relative values were adequate however, the more convenient Bio-Rad assay was used. No comparison has been made of the protein concentrations obtained from the two methods.

Figure 17: Standard curves for the estimation of protein by the method of

a) Bradford (1976)

b) Lowry et al (1951)


A
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE AT 595 nm
0
5
10
15
20
PROTEIN IN ASSAY (μg ml⁻¹)


B
0.3
0.2
0.1
0
ABSORBANCE AT 700 nm
0
25
50
75
100
PROTEIN IN SAMPLE (μg ml⁻¹)

4.3: Disruption methods

In order to optimise the yield and/or specific activity of α-GPDH from a given number of cells, several methods of cell disruption were attempted, the choice of which were based upon the work of Oduro et al. (1980a,b). Three of the treatments used were similar and are described together below:

a) incubation with 0.5% (w/v) Saponin
b) incubation with 0.5% (w/v) Triton X-100
c) incubation without detergent.

These treatments were carried out as follows: trypanosomes were collected from a DEAE-cellulose column and the buffer in which they were eluted spun off. The resulting pellet of whole cells was resuspended in 5ml disruption buffer with the treatment added where required. The disruption buffer comprised:

25.0mM Tris-HCl
1.0mM EDTA
0.5mM PMSF(added from a 100mM stock solution in acetone)
1.0mM DTT (added prior to use)
0.25M sucrose (added prior to use)
plus the relevant treatment added as a 5% (w/v) solution in 25mM Tris-HCl buffer
Final pH 7.8

The cells were incubated in the buffer for 30 minutes in ice with occasional gentle agitation and the procedure was then continued as in Figure 18a.

A fourth treatment involved the disruption of trypanosomes by grinding with silicon carbide (SiC) (Grade C6-F400, The Carborundum Co., Manchester, England) as shown in Figure 18b. The activities obtained at various stages are shown as Figure 19, each treatment being performed with the same number of trypanosomes. The most promising disruption method proved to be the grinding of the cells with SiC as an abrasive, the pellet from the 14,000 x g spin treated with Triton X-100 (14.5KP(T)) being the most active fraction. When several different 14.5KP(T) samples were run on 10% (w/v) SDS-polyacrylamide gels they were all seen to have a similar banding pattern but the relative intensities of the bands differed. In an attempt to 'standardise' the composition of the various extracts trypanosomes were ground with SiC in a Braun Mill homogeniser. A five second disruption was found to be adequate for no live cells to be visible microscopically. Prior to and following disruption of the cells the procedure was as in Figure 18b.

Samples were assayed at various stages of the purification for both α-GPDH activity and protein concentration, the results being compared with the same stage of a hand-ground extract, each sample initially containing the same number of trypanosomes. In each case the initial pellet of trypanosomes was resuspended in 2.5ml buffer/1 x 10^{10} cells and ground with 5g SiC/1 x 10^{10} cells. A summary of the results obtained is shown as Table 4.

It is apparent that the grinding of trypanosomes by hand with

Figure 18a: Scheme for the disruption of trypanosomes by detergents. Each initial trypanosome suspension contained 5×10^8 cells. Following centrifugation, the cell pellet was resuspended in 5ml disruption buffer (page 63) containing the appropriate detergent (0.5% (w/v) saponin or 0.5% (w/v) Triton X-100). The volumes and enzyme activities obtained at each stage of this scheme are shown in Figure 19.

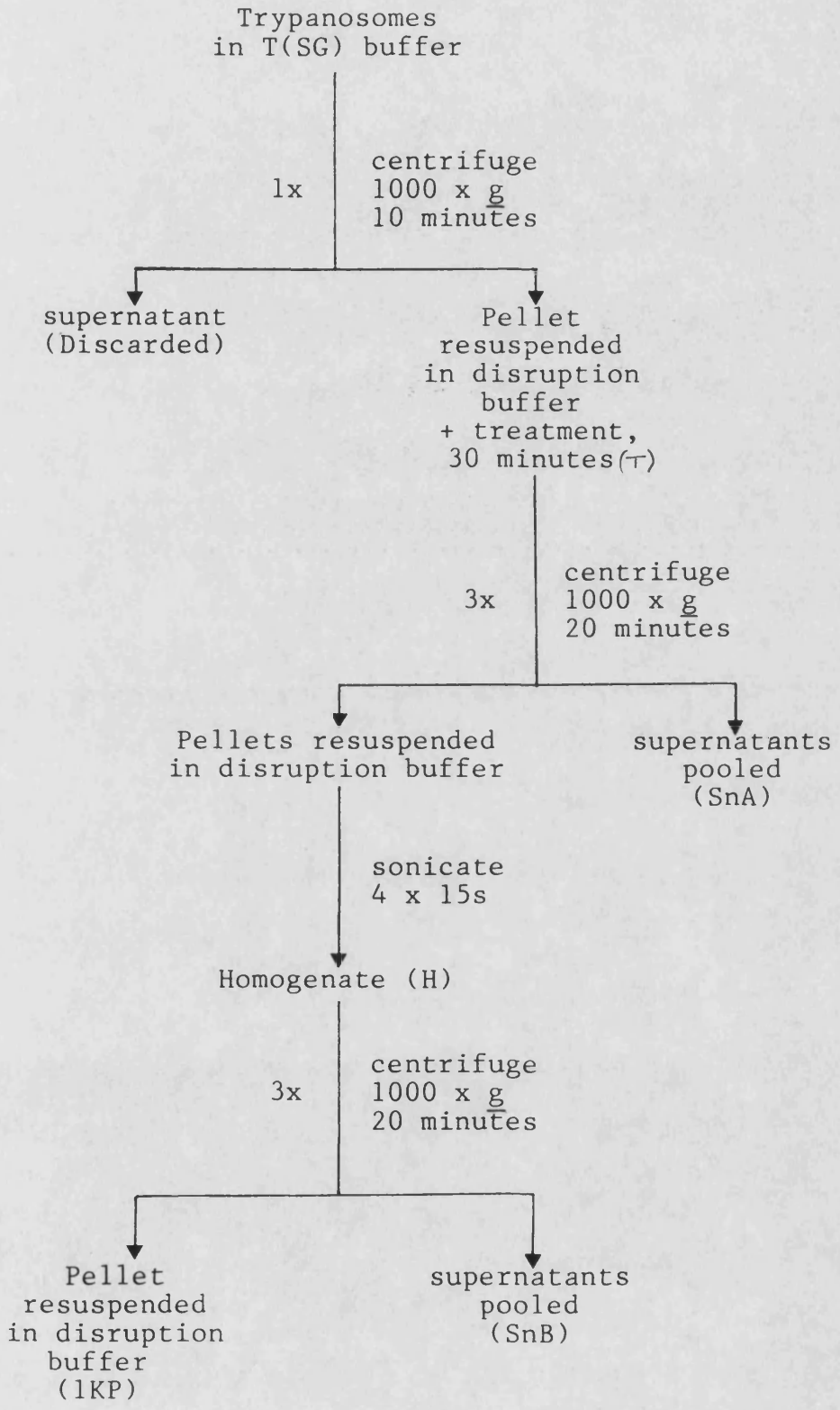

Trypanosomes
in T(SG) buffer
1x
centrifuge
1000 x g
10 minutes
supernatant
(Discarded)
Pellet
resuspended
in disruption
buffer
+ treatment,
30 minutes(T)
3x
centrifuge
1000 x g
20 minutes
Pellets resuspended
in disruption buffer
supernatants
pooled
(SnA)
sonicate
4 x 15s
Homogenate (H)
3x
centrifuge
1000 x g
20 minutes
Pellet
resuspended
in disruption
buffer
(1KP)
supernatants
pooled
(SnB)

Figure 18b: Scheme for the disruption of trypanosomes by grinding. The initial trypanosome suspension contained 5 x 10^8 cells. Following centrifugation, the cell pellet was resuspended in 2.5ml disruption buffer (page 63) and ground with 5g silicon carbide per 10^{10} cells. The volumes and enzyme activities obtained in each step are shown in Figure 19.

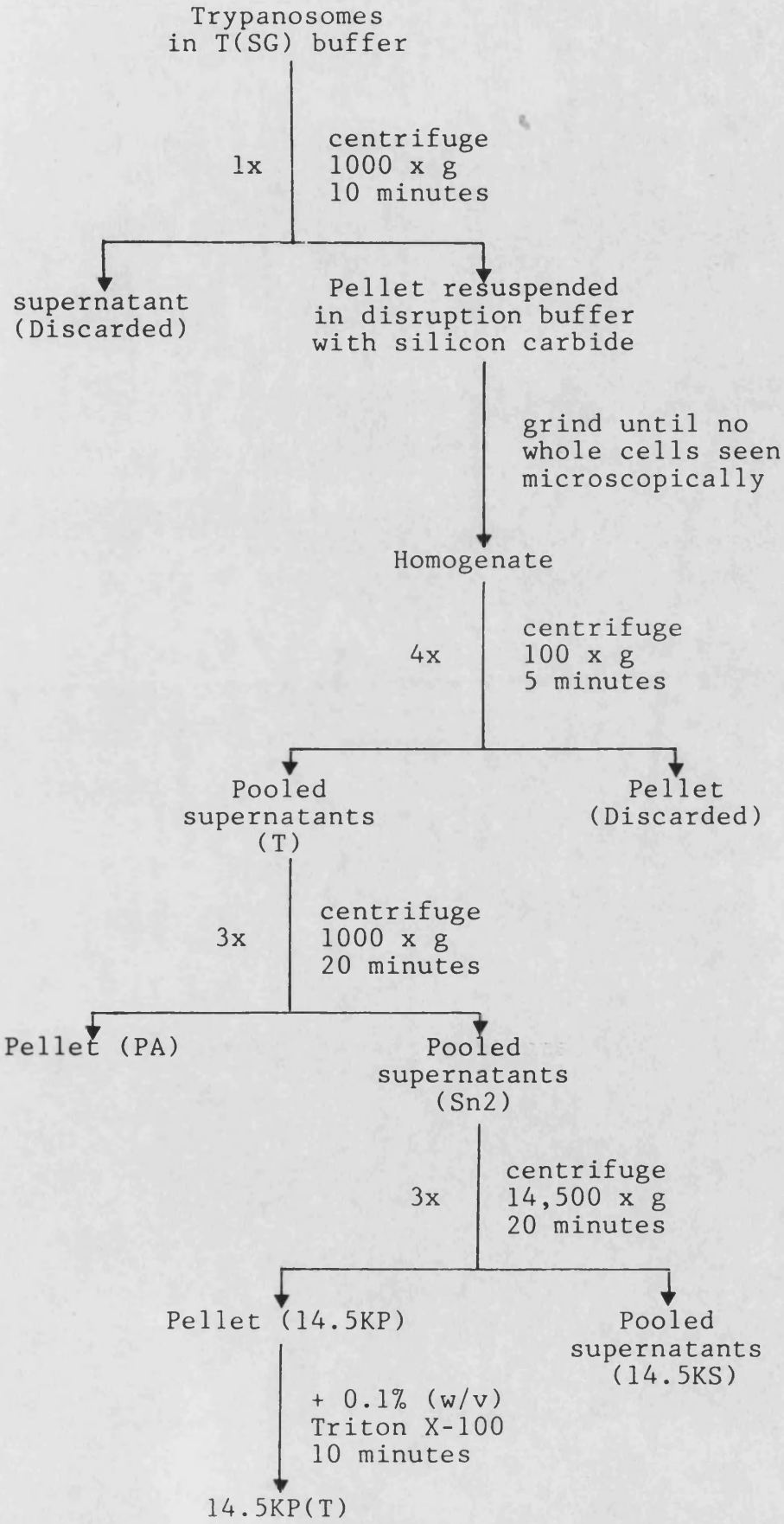

Trypanosomes
in T(SG) buffer
1x
centrifuge
1000 x g
10 minutes
supernatant
(Discarded)
Pellet resuspended
in disruption buffer
with silicon carbide
grind until no
whole cells seen
microscopically
Homogenate
4x
centrifuge
100 x g
5 minutes
Pooled
supernatants
(T)
Pellet
(Discarded)
3x
centrifuge
1000 x g
20 minutes
Pellet (PA)
Pooled
supernatants
(Sn2)
3x
centrifuge
14,500 x g
20 minutes
Pellet (14.5KP)
Pooled
supernatants
(14.5KS)
+ 0.1% (w/v)
Triton X-100
10 minutes
14.5KP(T)

Figure 19: Comparison of the yields and activity of α-GPDH from trypanosomes disrupted by various methods (see Section 4.3 for details). The abbreviations used for sample names may be referred to in Figures 18a and 18b.

Treatment 1 is 0.5% (w/v) saponin

Treatment 2 is 0.5% (w/v) Triton x-100

Treatment 3 contains no detergent

Treatment 4 is grinding with silicon carbide

Sample	Volume (ml)	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)
T1	5.0	11.90	8.75	59.50	43.75	1.36
T2	5.0	14.38	10.00	71.90	50.00	1.44
T3	5.0	4.41	8.60	22.05	43.00	0.51
T4	5.0	3.11	4.09	15.55	20.45	0.76
SnA1	8.12	2.94	3.38	23.87	27.44	0.87
SnA2	9.25	5.06	3.94	46.80	36.44	1.28
SnA3	9.88	0.00	0.74	0.00	7.31	0.00
H1	4.0	6.02	3.94	24.08	15.76	1.53
H2	4.0	4.59	3.82	18.36	15.28	1.20
H3	4.0	2.63	9.10	10.52	36.40	0.29
SnB1	9.07	2.53	1.96	22.95	17.78	1.29
SnB2	9.33	1.63	1.95	15.21	18.19	0.84
SnB3	8.80	1.00	3.60	8.80	31.68	0.28
1KP1	2.0	0.03	0.64	0.06	1.28	0.05
1KP2	2.0	0.38	0.80	0.76	1.60	0.48
1KP3	2.0	1.88	2.57	3.76	5.14	0.73
PA	2.0	0.33	1.24	0.66	2.48	0.27
14.5KS	15.56	0.33	1.84	5.13	28.63	0.18
14.5KP	3.0	1.75	1.96	5.25	5.88	0.89
14.5KP(T)	3.0	9.50	1.96	28.50	5.88	4.85

Table 4: Comparison of disruption of trypanosomes by grinding with silicon carbide by hand and with a Braun mill

Sample	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Hand-ground:				
T	20.5	34.64	45.92	0.75
P1	2.0	2.59	3.56	0.73
Sn2	27.7	32.56	40.23	0.81
14.5KS	38.6	5.59	33.67	0.17
14.5KP(T)	15.0	14.90	6.30	2.37
Braun-Mill				
T	23.0	29.03	51.56	0.56
P1	1.0	0.43	1.24	0.35
Sn2	30.7	25.02	38.16	0.66
14.5KS	46.8	8.87	29.23	0.30
14.5KP(T)	15.0	11.78	8.24	1.43

silicon carbide as an abrasive gives rise to a greater yield of α-GPDH activity and also a considerably higher specific activity. This, combined with the greater simplicity of the hand-grinding method led to its use for the preparation of all future 14.5KP(T) extracts.

4.4: Affinity chromatography

4.4.1: Dye-Ligand chromatography

The choice of affinity ligand was made after reference to the work of Watson et al. (1978), McGinnis (1983) and to the "Dye-Ligand Chromatography" handbook published by the Amicon Corporation, Mass., U.S.A. The Matrex Gel Blue A used comprised approximately 5% (w/v) cross-linked agarose covalently coupled via ether linkages to the triazinyl dye Cibacron Blue 3GA (Ciba-Geigy) at a concentration of 2.83mg/ml gel.

The gel was washed batchwise with 7M urea in 0.5M sodium hydroxide to remove any free dye and sodium azide preservative and then washed several times with 20mM triethanolamine buffer, pH 7.8 (running buffer). The gel was packed into a 1 x 8cm column in a controlled temperature room at 4°C and loaded with a 14.5KP(T) extract until α-GPDH activity appeared in the eluate. Excess (ie unbound) activity was then washed through with running buffer until no further activity was detectable in the eluate, and the quantity bound calculated.

Elution was then attempted with a linear gradient of 0-0.5M sodium chloride in running buffer, the gradient volume being 50ml and

fractions of 1ml collected. No activity was recovered with this system and so the same column was washed through with 1M NaCl and re-equilibrated with running buffer. The column was re-run with 1ml 14.5KP(T) applied, no α-GPDH activity being washed through the column with buffer. Elution was attempted with 10ml of each of the following combinations of substrates and co-factors, all applied manually:

1) 0.25mM DHAP + 0.3mM NAD^+

2) 4.0mM α-GP + 0.1mM NADH

3) 0.25mM DHAP + 0.1mM NADH

4) 4.0mM α-GP + 0.3mM NAD^+

Again no activity was recovered; consequently no further work was carried out with affinity columns of the dye-ligand type.

4.4.2: Chromatography using ATP-agarose

An 8-(6-aminohexyl)-amino-ATP-Sepharose affinity column was used by Lee et al. (1979) and Niesel et al. (1980) as a major step in the purification of α-GPDH from a mixture of enzymes from Drosophila. Cronin and Tipton (1985) used ATP-agarose Type 2 (P-L Biochemicals, Milwaukee, U.S.A.) in the purification of phosphofructokinase from T. brucei.

The buffer used for equilibration of the matrix was that described as Buffer A by Lee et al. (1979) (10mM potassium phosphate containing 1mM EDTA and 1mM DTT, pH 6.0). From the manufacturer's value of 1-5μmol ligand/ml of agarose and the assumptions that

trypanosomal α-GPDH has a molecular weight of approximately 78,000 and that 9.4% of the protein content of any 14.5KP(T) extract was α-GPDH (Aman et al., 1985), the calculated volume of ATP-agarose required for adsorption was too small to be measured for use. In fact 25μl of ATP-agarose Type 3 (equivalent to that used by Lee et al., 1979), incubated with 0.5ml 14.5KP(T) extract for 40 minutes in ice with frequent shaking was found to adsorb a maximum of 78% α-GPDH activity in the extract (Figure 20).

Following centrifugation at 15,000 x g for one minute, elution of α-GPDH activity from the pellet was attempted with several volumes of 1mM NADH in Buffer A, incubated together for 15 minutes in ice. Following centrifugation as above, the supernatant was assayed for enzyme activity (Table 5). It can be seen that recovery was poor, and elution was then attempted from the same pellet with 5mM DHAP and 2mM NADH in Buffer A.

The recovery of activity from the two elutions with NADH was 17% and from the DHAP/NADH elution, 14.5%. Both the recovery of activity and the increase in specific activity, where calculated, were acceptable for initial attempts at the technique. Further attempts proved unreliable however, and the potential expense of the large-scale ATP-agarose affinity chromatography led to discontinuation of its use.

4.4.3: Chromatography using AMP-Sepharose

The methods and principles employed were adapted from the work of Lowe et al. (1974a,b) and Harvey et al. (1974). Affinity chromatography

Figure 20: The adsorption of trypanosomal α-GPDH by ATP-agarose Type 3. A volume of 25 µl ATP-agarose Type 3 was incubated with 0.5ml 14.5KP(T) extract on ice with shaking. Samples were removed at intervals and, following brief centrifugation, the supernatant assayed for any α-GPDH activity which remained unbound. It can be seen that a maximum of 78% α-GPDH activity was bound.

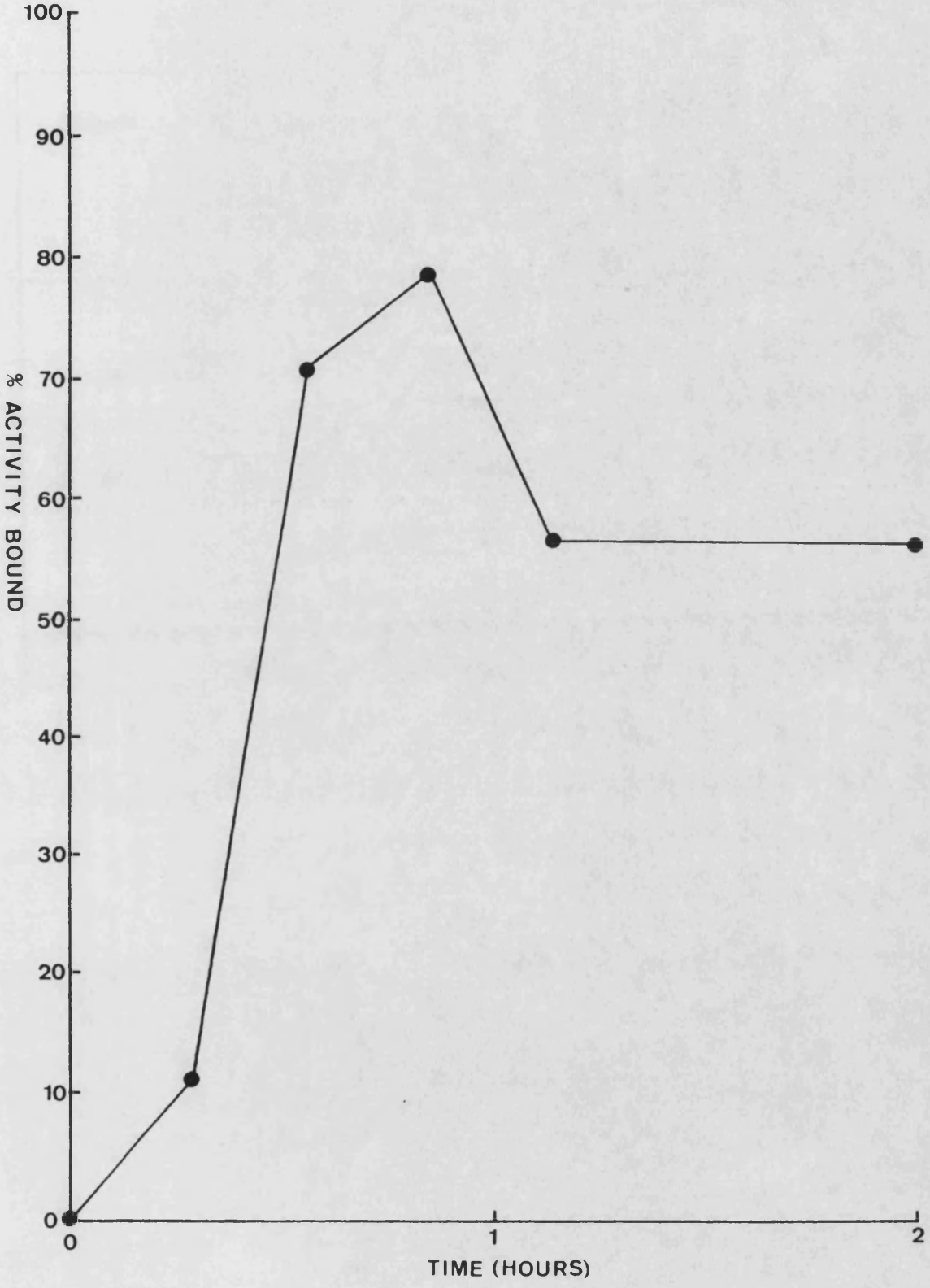

100
90
80
70
60
50
40
30
20
10
0
% ACTIVITY BOUND
0
1
2
TIME (HOURS)

Table 5: Affinity chromatography of α-GPDH from T brucei on ATP-agarose

Sample	Vol (µl)	α-GPDH activity (U/ml)	Protein content (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
14.5KP(T)	500	1.329	0.301	0.665	0.150	4.430
S'natant	525	0.212	0.169	0.111	0.089	1.247
1mM NADH	500	0.137	0.012	0.068	0.006	11.333
1mM NADH	500	0.090	0.007	0.045	0.0035	11.250
DHAP/NADH	200	0.479	NA	0.096	NC	NC

NA = not assayed.

NC = not calculated

was attempted with adenosine 5'-monophosphate-Sepharose 4B in which AMP was attached through an N6-amino group to Sepharose 4B with a 6-carbon spacer arm. The AMP-Sepharose was swollen and equilibrated as instructed by the manufacturers with 0.1M phosphate buffer at pH 7.0. The slurry was then centrifuged briefly in a Sarstedt microfuge and the supernatant discarded. A pellet of resin, of approximate mass 0.5g, was mixed with 0.5ml 14.5KP(T) extract and incubated for 30 minutes on ice with gentle agitation. Following brief centrifugation, as above, the supernatant was removed and the pellet washed twice with 0.1M phosphate buffer, pH 7.0. Elution of α-GPDH from the pellet was then attempted with 4ml volumes each of 5mM NADH and 5mM ATP but no activity was recovered. Stepwise elution was then carried out with NAD^+ in 0.1M phosphate buffer, pH 7.0, shown in Table 6. This method of elution was abandoned and elution with 0.1M phosphate buffers of increasing pH values was then attempted (Table 7). The method used was exactly as described above.

Due to the poor recoveries shown above no further investigation of affinity chromatography with AMP-Sepharose was carried out.

Table 6: Affinity chromatography of α-GPDH from T brucei with AMP-sepharose - elution with NAD^+

Sample	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	% recovery
14.5KP(T)	0.5	1.882	0.941	100
S'natant	0.5	0.274	0.137	14.6
Wash 1	1.0	0.000	0.000	0
Wash 2	1.0	0.000	0.000	0
Eluants:				
0.25mM NAD^+	0.5	0.168	0.084	8.9
0.50mM NAD^+	0.5	0.000	0.000	0
0.75mM NAD^+	0.5	0.000	0.000	0

Table 7: Affinity chromatography of α-GPDH from T. brucei - elution by increasing pH

Sample	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	% recovery
14.5KP(T)	0.5	1.922	0.961	100
S'natant	0.5	0.418	0.209	21.8
Wash 1	1.0	0.000	0.000	0
Wash 2	1.0	0.000	0.000	0
Eluants:				
pH 5.0	1.0	0.000	0.000	0
pH 6.0	1.0	0.161	0.161	16.75
pH 7.0	1.0	0.042	0.042	4.37
pH 8.0	1.0	0.000	0.000	0

4.5: Polyethylene glycol precipitation

The fractional precipitation of proteins with non-ionic water-soluble polymers such as polyethylene glycol (PEG) was first described by Polson *et al.* (1964), although these workers were unable to offer any explanation for the precipitating effect of PEG on protein solutions. Since then however, it has become apparent that the method is a steric exclusion mechanism whereby proteins are concentrated in the extrapolymer space, eventually exceeding their solubility limit under the given conditions (Ingham, 1984).

A range of PEG suspensions of concentrations between 6 and 100% (w/v) were prepared from PEG 8000 (formerly PEG 6000) in 20mM triethanolamine (TEA) buffer, pH 7.4. Equal volumes of 14.5KP(T) extract and the relevant PEG suspension were mixed to yield final PEG concentrations between 3 and 50% (w/v). The mixture was incubated for 45 minutes at room temperature with constant stirring, and then spun in a Du Pont Sorvall RC-5B Refrigerated Superspeed centrifuge at 36,900 x *g* for 40 minutes. Each supernatant was assayed for α-GPDH activity. The 'analytical precipitation curve' obtained is shown as Figure 21.

It can be seen that the most promising final concentration of PEG was 50% (w/v) and further investigation of the technique was carried out using this concentration, the method being repeated as above. In order that further work could be carried out on the enzyme, an attempt was made to adsorb the enzyme to, and elute it from, an anion-exchanger thus removing the extremely viscous PEG from the environment of the enzyme. To this end the PEG-containing pellet was

resuspended in 20mM TEA buffer pH 7.4, the volume used being approximately equal to that of the initial PEG and enzyme sample mixture. The resuspended pellet was then mixed with half its own volume of DEAE-Sephacel which had been equilibrated with 20mM TEA buffer pH 7.4 prior to use. After incubation for 25 minutes at room temperature with regular gentle agitation, the mixture was spun in a BTL bench centrifuge at 800 x g for 5 minutes. The supernatant was retained for assay. The DEAE-Sephacel pellet was resuspended in 2ml 0.2M sodium chloride in 20mM TEA buffer, pH 7.4, and incubated at room temperature for ten minutes before being centrifuged as above. The resulting pellet was resuspended in 3ml 0.2M sodium chloride in 20mM TEA buffer pH 7.4 and treated exactly as previously. This procedure is shown in Figure 22. The results obtained are shown in Table 8.

These initial results indicated that PEG precipitation may have been a useful technique once the method of elution had been improved. However use of this type of elution method meant that the choice of subsequent purification procedures would have been limited by the presence of sodium chloride at potentially high concentrations. Practical difficulties were encountered when working with PEG at the percentage required and these reasons combined led to the discontinuation of investigation of polyethylene glycol precipitation as a possible step in the purification of α-GPDH.

Figure 21: The 'analytical precipitation curve' for the precipitation of trypanosomal α-GPDH with PEG. Suspensions of PEG 8000 in 20mM triethanolamine buffer, pH 7.4, were prepared in the range 6 - 100% (w/v). Equal volumes of 14.5KP(T) extract and the relevant PEG suspension were mixed to yield final concentrations of between 3 and 50% (w/v) PEG. After 45 minutes incubation at room temperature and centrifugation at 36,900 x g for 40 minutes, the supernatant was assayed for free α-GPDH activity.

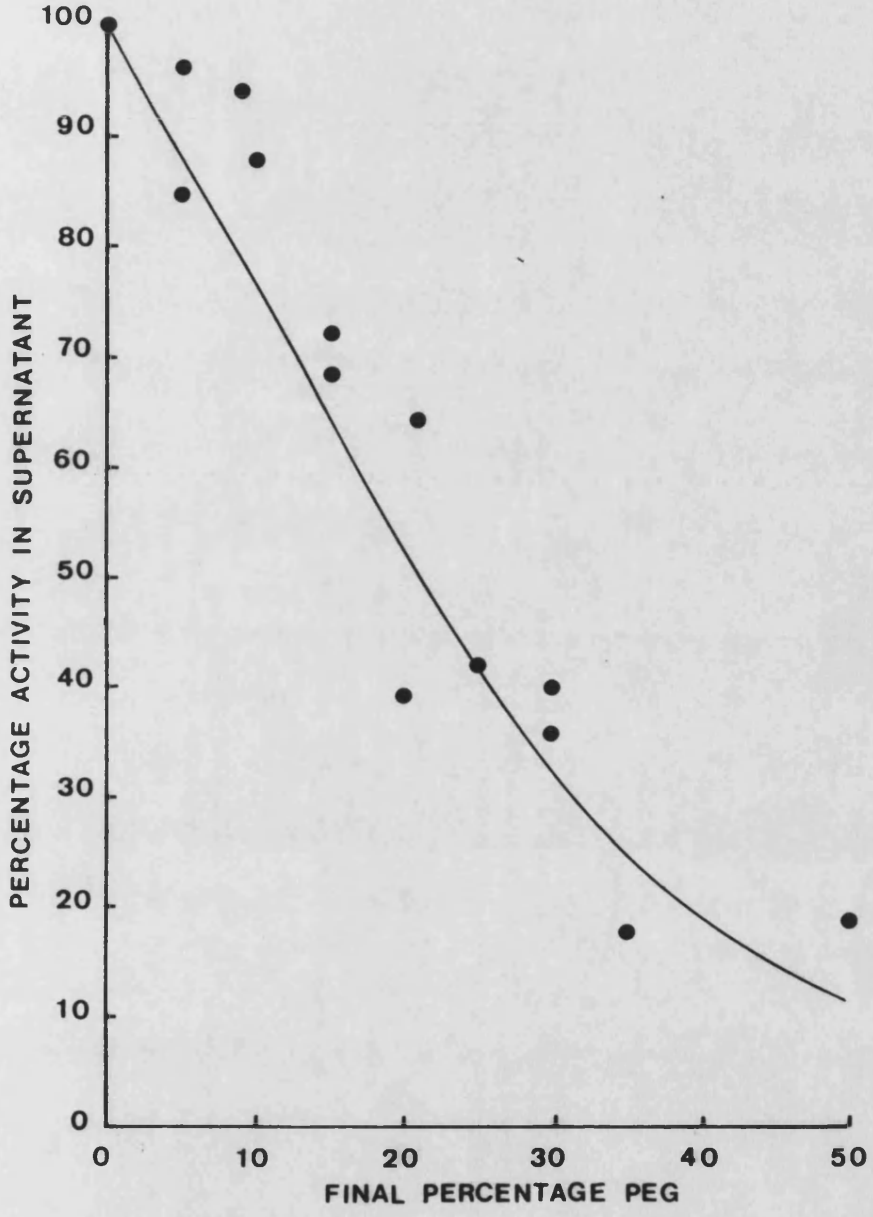

PERCENTAGE ACTIVITY IN SUPERNATANT
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
FINAL PERCENTAGE PEG

Figure 22: The attempted precipitation of α-GPDH from T. brucei with polyethylene glycol. The volumes used and enzyme activities obtained at each stage are shown in Table 8 on page 81.

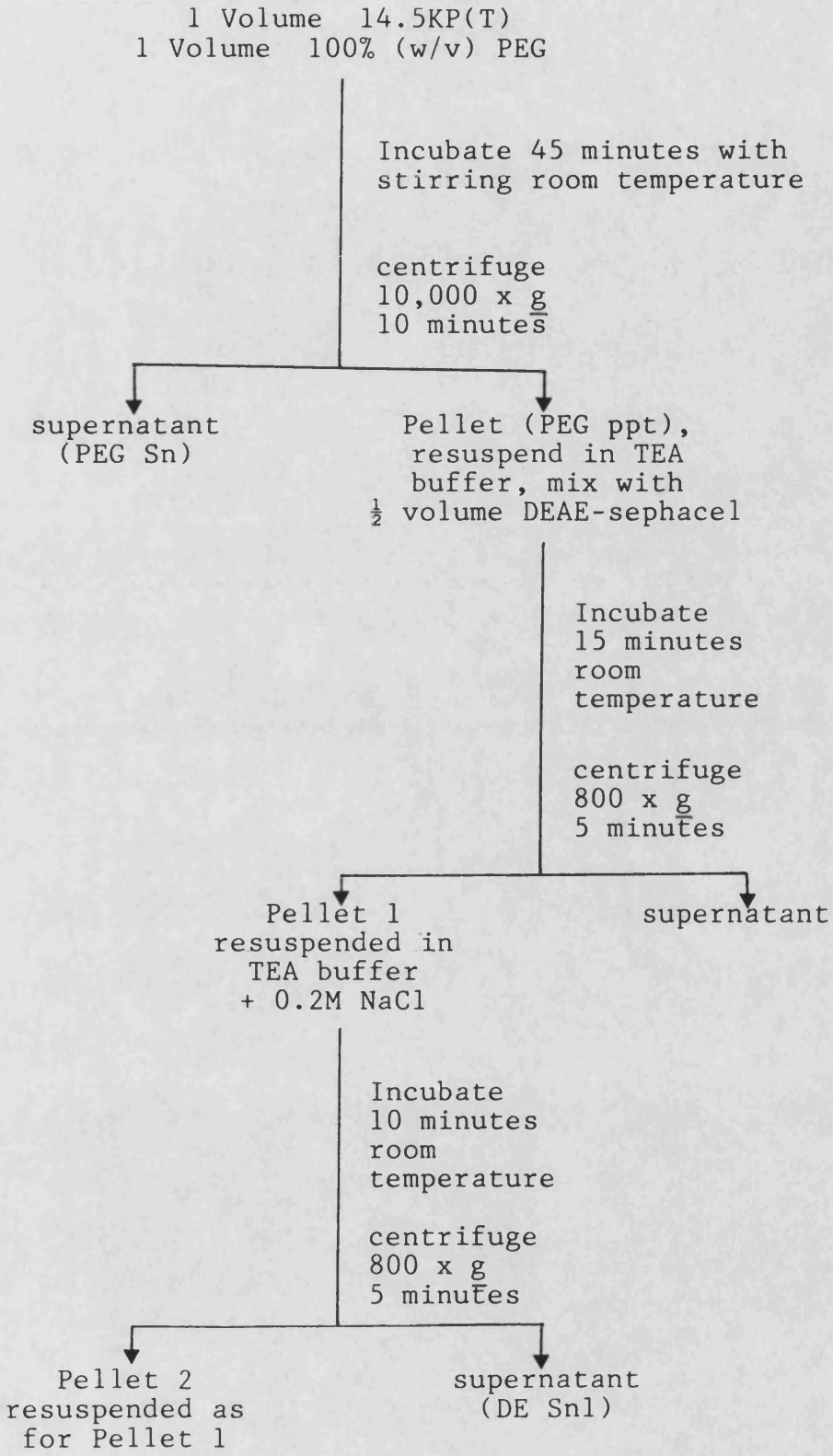

1 Volume 14.5KP(T)
1 Volume 100% (w/v) PEG
Incubate 45 minutes with stirring room temperature
centrifuge 10,000 x g 10 minutes
supernatant (PEG Sn)
Pellet (PEG ppt), resuspend in TEA buffer, mix with ½ volume DEAE-sephacel
Incubate 15 minutes room temperature
centrifuge 800 x g 5 minutes
Pellet 1 resuspended in TEA buffer + 0.2M NaCl
supernatant
Incubate 10 minutes room temperature
centrifuge 800 x g 5 minutes
Pellet 2 resuspended as for Pellet 1
supernatant (DE Sn1)

Table 8: Fractional precipitation of α-GPDH from T. brucei using polyethylene glycol

Sample	Volume (ml)	α-GPDH activity (U/ml)	Total activity (U)	% activity
14.5KP(T)	2	2.064	4.128	100.00
PEG sn	4	0.026	0.104	2.52
PEG ppt	4	0.740	2.960	71.70
DE sn1	6	0.064	0.386	9.34
DE sn2	4	0.162	0.648	15.70
DE sn3	4	0.028	0.112	2.71

4.6: Heat precipitation

A sample of a 14.5KP(T) extract was diluted five-fold with Tris-HCl buffer pH 7.8 containing 1mM EDTA. Equal volumes of this solution were placed into glass test tubes, which were identical so that heat diffusion effects would be similar, each containing a small stirring-bar. To one sample were added NAD^+ and α-GP each to a final concentration of 1mM, these being substrates for α-GPDH which may have a stabilising effect on the enzyme during the treatment (Dixon and Webb, 1979). Each tube was placed into a water-bath which had been pre-heated to the desired temperature and which contained an immersible magnetic stirrer leading to continuous agitation of the samples. The stated period of immersion was strictly timed from the moment the enzyme solution itself reached the prescribed temperature.

After the required period of incubation the tubes were placed in ice until the temperature of the solution had fallen to approximately 4^oC, when the sample was centrifuged in an Ole Dich refrigerated microfuge for five minutes at 15,000 x g. The supernatants were assayed for both α-GPDH activity and protein content. Initial results seemed promising (see Table 9).

Repeated attempts at the purification of trypanosomal α-GPDH by heat precipitation proved inconsistent however, the precise conditions required for each treatment being difficult to reproduce. Due to the unreliability of this method it was not investigated further.

Table 9: Attempted purification of α-GPDH from T. brucei by heat precipitation

Sample	Treatment	Specific activity (U/mg)	% recovery
14.5KP(T) (=A)	Diluted 1/5	2.314	122.6
14.5KP(T) (=B)	+ 1mM NAD^+, 1mM α-GP	1.888	100.0
As B	10 min at 40°C	1.907	67.7
As B	20 min at 40°C	1.483	51.6
As B	10 min at 50°C	2.645	61.3
As B	10 min at 60°C	5.359	71.0
As B	20 min at 60°C	5.490	67.7
As B	10 min at 70°C	0.000	0.0

4.7: Gel filtration

For the attempted separation of α-GPDH from 14.5KP(T) extracts by the technique of gel filtration, various conditions were used as shown in Table 10. All gels used were either Sephadex G-100 or G-200.

Enzyme extracts containing 10% (w/v) sucrose were carefully loaded onto the column before elution was started. The void volume of each column was determined by the use of Dextran Blue as an excluded marker. The α-GPDH activity recovered from columns 1 to 3 was eluted in the void volume of the columns. This may have been due to the aggregation of the glycosomal enzymes under conditions of low ionic strength (Misset and Opperdoes, 1984), the use of a buffer with a higher ionic strength possibly promoting the dissociation of any such complex and individual behaviour of each enzyme. To this end a phosphate buffer with an ionic strength of 0.2M (I = 0.2M P) was introduced, prepared as described by Long (1961).

Following this change of buffer the enzyme was still eluted in the void volume of column 3, a column of Sephadex G-100. Columns 4 to 9 however contained Sephadex G-200 which had a larger pore size, and the enzyme activity was not eluted in the void volume but the percentage of α-GPDH activity recovered decreased. The recovery of protein from column 8 was 92% of that applied. The recovery of activity and protein from column 6 is shown in Figure 23.

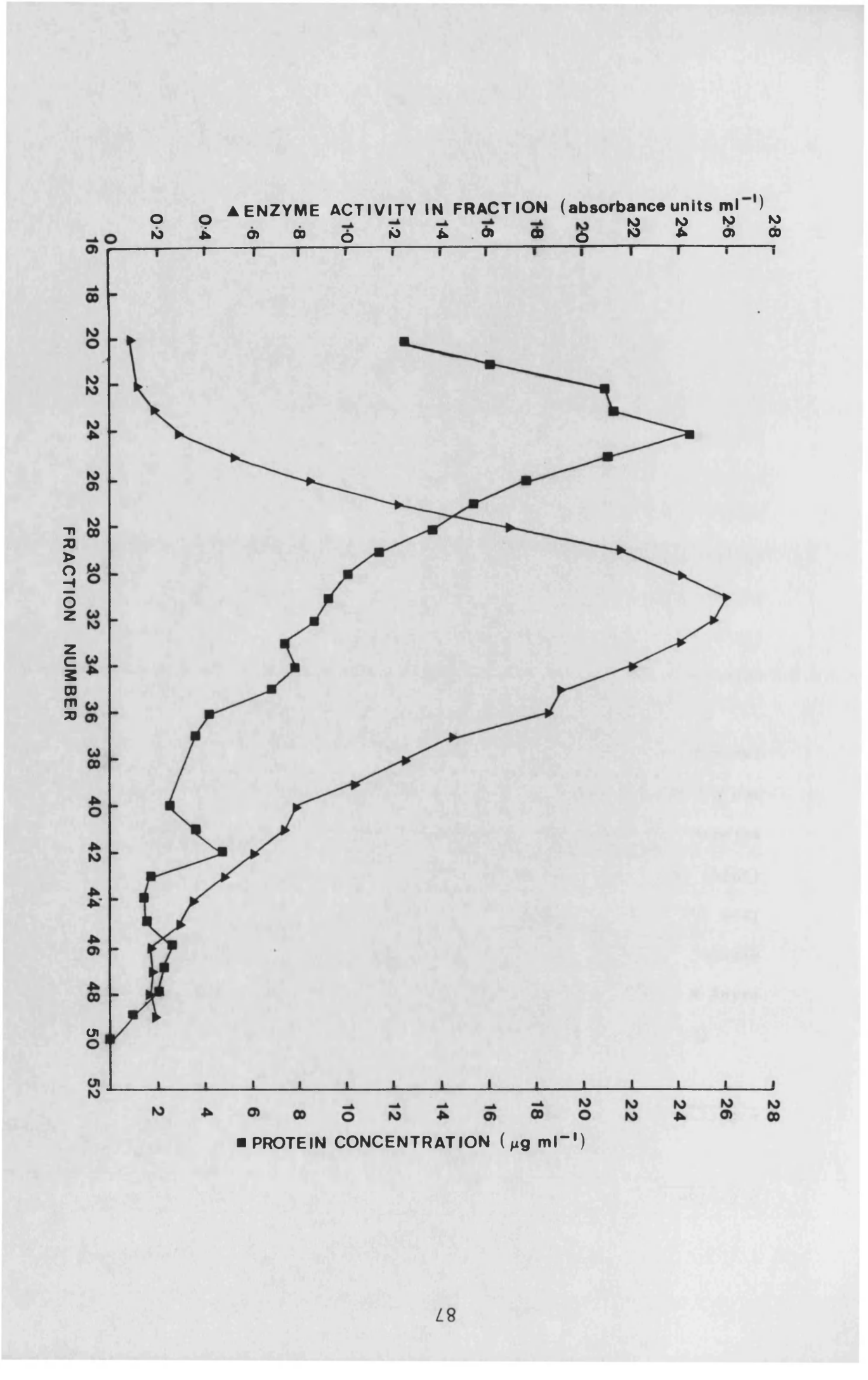
The possibility that the high ionic strength of the elution buffer had caused the dissociation of both a multi-enzyme complex and any subunit structure of the enzyme itself was investigated. The ionic strength of the environment of the enzyme was reduced by passing

fractions from column 6 through a small Sephadex G-25 column, eluting with 50mM phosphate buffer containing 8mM $MgCl_2$, pH 7.4. No activation of α-GPDH activity was evident upon assay of the eluates from this column. The inclusion of DTT in the elution buffer of column 9 did not improve the low recovery of activity and the investigation of gel filtration as a purification procedure was consequently discontinued.

Table 10: Gel filtration of α-GPDH from T. brucei

Gel type	Column size (cm)	Buffer	Buffer pH	% recovery α-GPDH activity
1) G-200	38 x 2.0	20mM TEA	7.8	27.5
2) G-100	27 x 0.9	20mM TEA	7.8	76.5
3) G-100	27 x 0.9	I = 0.2M P	7.4	60.1
4) G-200	35 x 2.5	I = 0.2M P	7.4	3.8
5) G-200	35 x 2.5	I = 0.2M P	7.4	3.2
6) G-200	24 x 2.0	I = 0.2M P + 1mM EDTA	7.4	47.3
7) G-200	74 x 1.5	I = 0.2M P + 1mM EDTA	7.4	0.0
8) G-200	35 x 2.5	I = 0.2M P + 1mM EDTA	7.4	15.4
9) G-200	37 x 2.5	I = 0.2M P + 1mM EDTA + 3mM DTT	7.4	13.1

Figure 23: The recovery of α-GPDH activity and protein after gel filtration. A 1ml volume of 14.5KP(T) extract was applied to a column of Sephadex G-200, 24.0 x 2.0cm in size. The column was equilibrated with phosphate buffer of 0.2M ionic strength with 1mM EDTA, pH 7.4, and was eluted with the same buffer. Fractions of 1.15ml were collected and a suitable volume of each assayed for α-GPDH activity. Of the applied α-GPDH activity, 47.3% in total was recovered from the column and the fraction with the highest specific activity (fraction 36) showed an increase of 2.1-fold over the applied extract.


▲ENZYME ACTIVITY IN FRACTION (absorbance units ml⁻¹)
0·2
0·4
·6
·8
1·0
1·2
1·4
1·6
1·8
2·0
2·2
2·4
2·6
2·8
FRACTION NUMBER
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
52
■ PROTEIN CONCENTRATION (μg ml⁻¹)
2
4
6
8
10
12
14
16
18
20
22
24
26
28

4.8: Ion-exchange chromatography

4.8.1: Fast Protein Liquid Chromatography

The separation of α-GPDH from a 14.5KP(T) extract was attempted using a mechanised Pharmacia Fast Protein Liquid Chromatography (FPLC) system. Previous work such as that by Bacchi et al.(1974) on α-GPDH from the trypanosomatid Crithidia fasciculata and Lambros and Bacchi (1976) on α-GPDH from Leptomonas species suggested that the use of an anion-exchanger would be appropriate and consequently Mono-Q HR 5/5 and Polyanion SI HR 5/5 pre-packed FPLC columns were used as recommended by the manufacturers.

For FPLC work with the Mono-Q column, Cl^- in the form of sodium chloride was chosen as the counter-ion in a 0 to 0.5M linear gradient, total volume 30ml in 20mM triethanolamine buffer, pH 7.4. No α-GPDH activity was eluted however and so the effect of sodium chloride (NaCl) on the enzyme was investigated: 100μl aliquots of a 14.5KP(T) sample were incubated on ice with 100μl volumes of NaCl in 20mM TEA buffer pH 7.4 over a range of concentrations. After 40 minutes incubation, 50μl samples were removed and assayed for α-GPDH activity. The results obtained are shown below.

Table 11: The effect of sodium chloride on the activity of α-GPDH from T. brucei

Concentration of NaCl in incubation	α-GPDH activity (U/ml)
500	0.381
250	0.378
125	0.386
50	0.369
25	0.357
0	0.351

It is apparent that any inhibition by NaCl is reversible. The trace produced at 280nm of the eluate from the Mono-Q column showed few significant protein peaks which, coupled with the evidence above, could show that the enzyme had been retained on the column. A further attempt at ion-exchange with the FPLC system was made using a Polyanion SI HR 5/5 column, which is a weaker anion-exchanger than the Mono-Q column. The elution gradient was 0 to 0.5M NaCl in 50mM Tris-HCl buffer, pH 8.0, again with a total volume of 30ml. The recovery of α-GPDH activity was 18% of that applied. The activity remaining on the column was subjected to attempted elution with 0.5M sodium sulphate in 50mM Tris-HCl buffer pH 8.0. Although sulphate was recommended by the manufacturers as a more efficient counter-ion than

chloride, no further activity was recovered.

4.8.2: Batchwise ion-exchange chromatography

Ion-exchange chromatography using small DEAE-Sephacel columns and manually-applied stepwise gradients of NaCl yielded poor recoveries of activity of between 4 and 15%. The use of DEAE-Sephacel batchwise seemed to be more useful: initially DEAE-Sephacel was equilibrated in 5mM phosphate buffer, pH 6.5, and a volume of the resulting slurry added to a suitable volume of 14.5KP(T) extract. The mixture was incubated on ice for 15 minutes with agitation and then centrifuged at 800 x g for three minutes. The supernatant was assayed for α-GPDH activity and if a significant proportion of the activity applied remained in the supernatant, further DEAE-Sephacel was added and the procedure repeated. Phosphate was chosen as the counter-ion to elute α-GPDH activity from the pellet, elution being stepwise from 50 to 500mM phosphate buffer, pH 8.0. A typical result appeared as in Table 12.

Combination of the three most active eluate fractions (25, 50 and 100mM phosphate) yielded 18.6% of applied protein and 51.8% of applied activity, giving an increase in specific activity of 2.8-fold. Whilst this, as a typical result, was encouraging, it was discovered that a maximum of 80% of the activity applied was adsorbed by the pellet. The possibility that two enzymes were present or that one enzyme had been proteolysed into two active fragments was then investigated.

The cation-exchanger CM-cellulose was equilibrated with 10mM phosphate buffer, pH 8.1, and DEAE-cellulose equilibrated as before.

Table 12: Adsorption to and elution from DEAE-sephacel of trypanosomal α-GPDH

Sample	Total activity (U)	%	Total protein (μg)	Specific activity (U/mg)
14.5KP(T)	2.760	100.00	225.75	12.22
S'natant	0.570	20.65	15.00	
Eluants:				
25mM	0.480	17.40	16.12	29.78
50mM	0.480	17.40	14.88	32.26
100mM	0.469	16.99	11.06	42.40
200mM	0.225	8.15	13.12	17.15
500mM	0.150	5.43	27.19	5.52

DEAE-cellulose and CM-cellulose were used in order that the ion-exchangers both had the same support so that a valid comparison could be made. The extent of adsorption of α-GPDH activity was discovered as described below. Firstly the amount of each slurry which gave maximal adsorption was determined experimentally. A sample of 14.5KP(T) extract was then incubated with this volume of DEAE-cellulose slurry, incubated and centrifuged as previously described. The supernatant was assayed for α-GPDH activity and then added to the optimum volume of CM-cellulose slurry. The incubation procedure was repeated and the supernatant again assayed for α-GPDH activity. The experiment was then carried out with the first ion-exchanger used being CM-cellulose and adsorption was also attempted with each ion-exchanger separately. The results obtained are shown below.

Table 13: The adsorption of α-GPDH from T. brucei to various ion-exchangers

Ion-exchanger(s)	Total α-GPDH activity adsorbed (%)
DEAE- then CM-cellulose	99.34
CM- then DEAE-cellulose	99.36
CM-cellulose only	59.04
DEAE-cellulose only	76.47

The sum of the activity adsorbed by DEAE-cellulose and CM-cellulose separately was expected to be approximately 99% but was

in fact 135.51%. Further experiments were carried out with the inclusion of microgranular cellulose (ie the matrix of both ion-exchangers) as an adsorbant. All three resins were equilibrated in 25mM Tris-HCl buffer, pH 7.8 and the following results obtained:

Table 14: Adsorption of α-GPDH from T. brucei to various ion-exchangers and their matrix

Ion-exchanger	Total α-GPDH activity adsorbed (%)
CM-cellulose	55.6
DEAE-cellulose	76.7
Cellulose support only	46.4

Estimation of the protein content of each supernatant showed that protein had been adsorbed and the enzyme not merely inactivated. A 14.5KP(T) extract was prepared in the following disruption buffer:

Tris-HCl	25.0mM
PMSF	0.5mM
DTT	1.0mM
EDTA	1.0mM
Sucrose	0.25M
Leupeptin	10μg/ml
Antipain	20μg/ml
Final pH	8.0

and the experiment repeated. The inclusion of the protease inhibitors leupeptin and antipain did not decrease the extent of adsorption to CM-cellulose or cellulose nor did it improve the percentage adsorption of α-GPDH to DEAE-cellulose however, and finally the method was abandoned.

4.9: Ammonium sulphate precipitation

A 14.5KP(T) sample was diluted 1/5 with disruption buffer (25mM Tris-HCl buffer containing 0.5mM PMSF, 1.0mM DTT, 1.0mM EDTA and 0.25M sucrose, pH 7.8) and glycerol was added to a final concentration of 10% (v/v). Ammonium sulphate precipitation was carried out sequentially on this sample. The mass of solid ammonium sulphate required to bring the sample to the first 'cut' (Dawson et al., 1969) was added slowly to the sample which was incubated on ice for a further 15 minutes with continuous gentle stirring. The sample was then spun at 12,000 x g for 10 minutes in an MSE-18 High Speed centrifuge. The pellet was resuspended in 2ml disruption buffer and 'desalted' on a small Pharmacia Sephadex G-25 gel filtration column, being eluted with disruption buffer. The eluant was then assayed for α-GPDH activity and protein concentration. The decanted supernatant was also assayed for both α-GPDH activity and protein content, following which sufficient solid ammonium sulphate was added to bring the ammonium sulphate concentration to that of the next 'cut' and the procedure repeated as above until the highest ammonium sulphate concentration required had been reached. The results obtained are shown in Table 15 from which it can be seen that the total protein recovery was 98.4% of that of the original sample while only 21.1% of the α-GPDH activity remained. That the loss of enzyme activity was due to the presence of ammonium sulphate was shown by the assay of a 14.5KP(T) sample in the presence of various concentrations of ammonium sulphate. The ammonium sulphate solutions were prepared in 50mM phosphate buffer, pH 7.4, and the volume of assay buffer adjusted to

Table 15: Ammonium sulphate precipitation of α-GPDH from T. brucei

Sample	α-GPDH activity (U/ml)	Volume (ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)
Diluted 14.5KP(T)	0.360	10.0	3.6	0.242	2.42
Resusp. pellets:					
0-20%	0	3.4	0	0.075	0.255
20-40%	0	3.6	0	0.050	0.180
40-60%	0.050	3.4	0.170	0.196	0.666
60-70%	0.076	3.6	0.274	0.170	0.595
70-80%	0.050	3.5	0.175	0.190	0.665
Final sn	0.014	10.0	0.140	0.002	0.020

compensate for their presence. The rates obtained are shown as Table 16:

Table 16: The effect of ammonium sulphate on the activity of α-GPDH from T. brucei

Concentration AS added to assay (% (w/v))	Final concentration AS in assay (% (w/v))	Enzyme activity (U/ml)	% activity
0	0	1.12	100.0
60	6	0.16	14.3
80	8	0.08	7.1

A similar experiment was carried out with the enzyme being assayed in the presence of ammonium sulphate at a final concentration of 4% (w/v) and also in the presence of potassium chloride (KCl) at the same final ionic strength (see Table 17).

It was apparent that the pH of the assay mixture may influence the amount of α-GPDH activity lost and assays were therefore carried out with ammonium sulphate added as a solution in buffer as before, the solution being adjusted to pH 7.4 (Table 18). Although the pH of the solution obviously did have some effect on the activity of the enzyme, it was not the most important factor in the effect of ammonium sulphate on α-GPDH activity. It was decided that ammonium sulphate

precipitation would not be included in the purification scheme for trypanosomal α-GPDH and also that the effect of the presence of salts on the activity of the enzyme would be investigated further.

Table 17: The effect of ionic strength and pH on the activity of trypanosomal α-GPDH

Sample	Enzyme activity (U/ml)	% activity	Final pH in assay
14.5KP(T)	1.29	100	7.4
14.5KP(T) + amm. sulphate	0.23	17.8	6.7
14.5KP(T) + KCl	0.35	27.1	7.0

Table 18: The effect of ionic strength on the activity of trypanosomal α-GPDH

Sample	Concentration AS in assay	Enzyme activity (U/ml)	%	Final pH in assay
14.5KP(T)	0	1.200	100	7.4
14.5KP(T) + AS	4	0.672	56	7.4

4.10: The effect of salts on α-GPDH activity

The effect of various ions on α-GPDH activity was investigated by the inclusion of solutions of these ions in the assay system. The solutions were each prepared in 20mM triethanolamine buffer and brought to pH 7.4. The volume of assay buffer was adjusted as necessary to maintain a constant total volume in the cuvette. The α-GPDH activity recorded, relative to that of the enzyme with no salts present in the assay system, is plotted in Figure 24 versus the final ionic strength of each salt in the assay.

Ionic strength is calculated thus: $I = \leq 1/2(c \times z^2)$ where c is the concentration of the salt (molar) and z is the charge on each ion. The ionic strength of a 3M solution of ammonium sulphate for example is $1/2(3 \times 2 \times 1) + 1/2(3 \times 4) = 3 + 6 = 9M$. An ammonium sulphate concentration of 3M is approximately equal to a 60% (w/v) solution. It can be seen from Figure 24 that the presence of such a concentration of ammonium sulphate in a sample to be assayed would have a considerable inhibitory effect. This experiment confirmed that ammonium sulphate precipitation would be of no use as a step in the purification of trypanosomal α-GPDH.

4.11: Polyacrylamide gel electrophoresis

Several methods of polyacrylamide gel electrophoresis were employed, the pH of buffers and the pore-size of the gel (ie concentration of acrylamide) being varied as required (Gordon, 1975; Hames and Rickwood, 1981). The basic methods however were those of

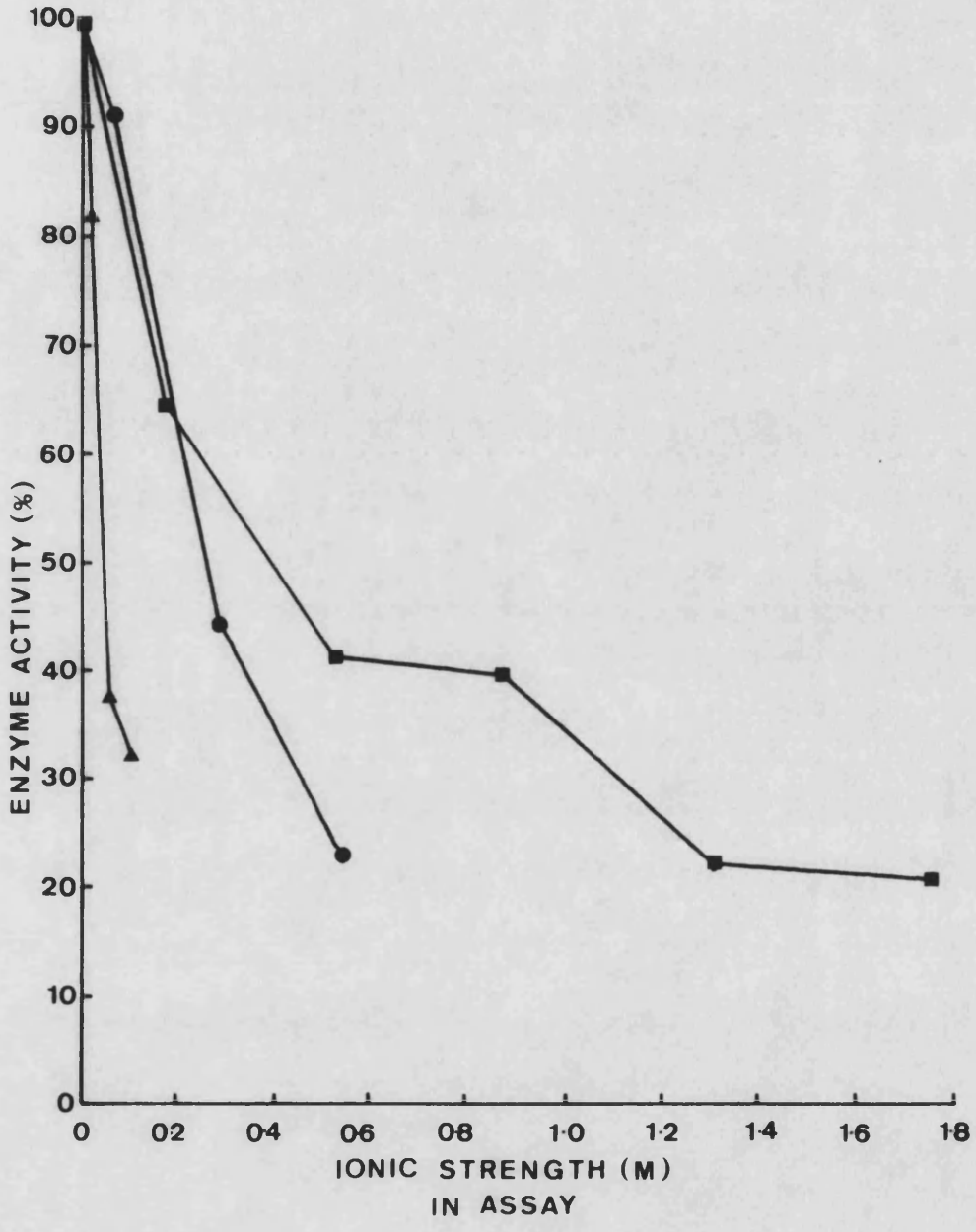
Figure 24: The effect of ionic strength on the activity of trypanosomal α-GPDH. The salts used were:

■ ammonium sulphate

▲ sodium sulphate

● ammonium chloride

The salt solutions were prepared in 20mM triethanolamine buffer and the final pH of each solution was 7.4. The activity of α-GPDH in the presence of various concentrations of each salt was determined and calculated as a percentage of the activity in the absence of added salts. This percentage was plotted versus the calculated ionic strength of the relevant salt in the cuvette.


100
90
80
70
60
50
40
30
20
10
0
ENZYME ACTIVITY (%)
0
02
04
06
08
1·0
1·2
1·4
1·6
1·8
IONIC STRENGTH (M)
IN ASSAY

Davis (1964) for non-denaturing electrophoresis and of Laemmli (1970) for electrophoresis in the presence of sodium dodecyl sulphate (SDS).

4.11.1: Sample preparation

Prior to application to the gels, a volume of the sample estimated to contain up to 50μg protein was either reduced to dryness with a vacuum pump or precipitated by 25% (w/v) trichloroacetic acid and washed twice with ethanol. For the electrophoresis of native proteins, samples were resuspended in a solution of 10% (w/v) sucrose and, as a tracking dye, 0.001% (w/v) bromophenol blue. For samples applied to SDS-polyacrylamide gels the dissolving buffer was as follows:

3M Tris-HCl buffer, pH 8.9	0.33ml
10% (w/v) SDS solution	1.00ml
Glycerol	1.00ml
2-mercaptoethanol	0.10ml
Distilled water	7.57ml
Bromophenol blue	0.10mg

Following resuspension of samples in this buffer they were immersed in a boiling water-bath for two minutes to ensure complete reduction of disulphide bonds by mercaptoethanol and denaturation of the protein mixture by the ionic detergent SDS.

4.11.2: Fixing and staining of gels with Coomassie blue

Gels were immersed in fixing solution (methanol:glacial acetic acid:distilled water in the ratio 5:1:5) and incubated at approximately 34°C for a minimum of one hour. The gels were then immersed in a solution of Coomassie Brilliant Blue R250 (1g/litre of fixing solution) for 45 minutes at 34°C. The gels were placed in destaining solution (methanol:glacial acetic acid:distilled water in the ratio 1:1.5:1.75) and agitated until all excess dye had been removed and bands of stained protein were visible. Gels were stored in distilled water until a permanent record had been made.

4.11.3: A sensitive method for the staining of protein

Very small amounts of protein were detected by "silver-staining" initially using the method of Morrissey (1981) and latterly by the method of Nielson and Brown (1984), this being the quicker and more sensitive. Neither stain was suitable for use on cylindrical gels.

4.11.4: Staining for α-GPDH activity

Having determined that the activity of α-GPDH was not destroyed by preparation of a sample for non-denaturing electrophoresis, some disc gels were stained for enzyme activity by the simultaneous capture technique described by Dewey and Conklin (1960) and Gabriel (1971). In this method, the NADH produced by the α-GPDH-catalysed reaction

reduced a tetrazolium dye to its insoluble coloured form, the dye therefore being deposited only where α-GPDH activity was present. In fact, NADH reduces tetrazolium dyes to a small extent only and so an auxilliary reaction was included whereby 5-methyl-phenazinium sulphate (PMS) was reduced and this in turn reduced the tetrazolium dye. The tetrazolium dye used was nitroblue-tetrazolium chloride (NBTC). The overall reaction is described by Mollering et al. (1974). The stain was, per gel:

10mM NAD^+	140µl
0.1M α-GP	200µl
NBTC	2mg
PMS	0.7mg
MET8 buffer *	6.66ml

* 20mM Tris-HCl, 1mM EDTA, 10mM $MgCl_2$, pH 8.0

The gels were placed into the staining solution, left in darkness at room temperature for approximately 20 minutes until a blue-black coloration had developed and then washed with 7.5% (v/v) acetic acid and stored in distilled water.

4.11.5: Staining for activity of a multi-enzyme complex

The method for this stain was adapted from the basic assay system of Oduro et al. (1980b). The dyes used were as above and therefore required production of NADH in the reaction. The system of Oduro

et al. (1980b) was designed to assay the reaction shown in Figure 25a. By the omission of iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the inclusion instead of sodium arsenate to prevent any reversal of the GAPDH-catalysed reaction, the reaction occurring became that shown in Figure 25b. The reaction mixture was made up in MET8 buffer as follows:

ATP	0.94mM
$MgCl_2$	13.90mM
NAD^+	0.40mM
Sucrose	0.25 M
Glucose	0.40 M
Sodium arsenate	10.00mM
PMS	0.1mg/ml
NBTC	0.3mg/ml

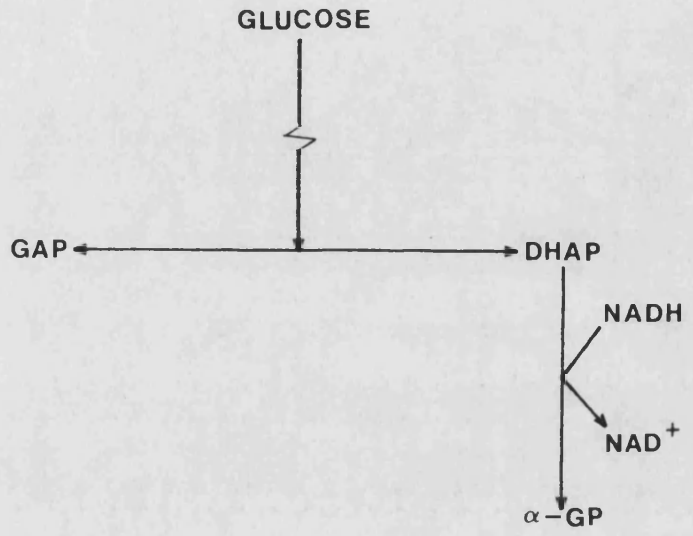
A control stain containing only NAD^+ and dyes was carried out to visualise any reduction of NAD^+ by means other than the complex-catalysed reaction pathway.

4.11.6: Gels run

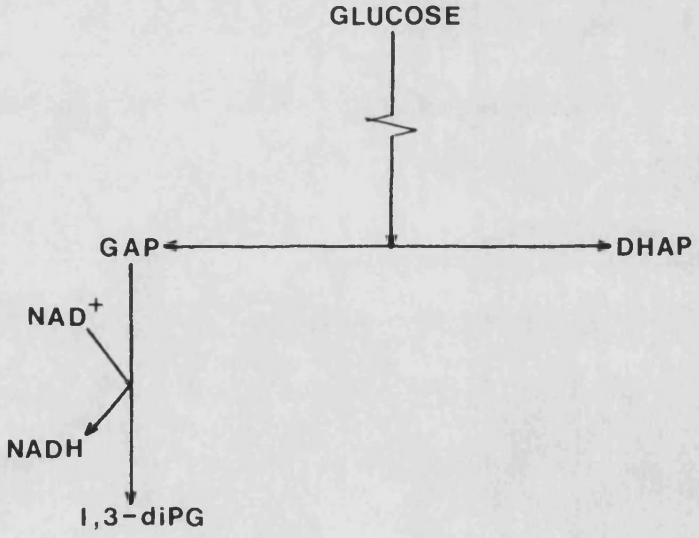
When applied to non-denaturing gels no 14.5KP(T) sample penetrated more than 0.5cm into the gel. Gels were made at 3% (w/v) acrylamide plus 0.5% (w/v) agarose but no increase in penetration was seen. The gels were stained for α-GPDH activity and the presence of a multi-enzyme complex and both were tentatively identified.

Figure 25: The reactions occurring in the assay of a multi-enzyme complex. The reaction shown in a) was used by Oduro et al. (1980b) while that in b) was devised during the work presented here. The system used by Oduro et al. included iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) while the system shown in Figure 25b instead contained sodium arsenate to prevent reversal of the GAPDH-catalysed reaction now able to take place.

A


GLUCOSE
GAP
DHAP
NADH
NAD+
α-GP

B


GLUCOSE
GAP
DHAP
NAD+
NADH
1,3-diPG

Throughout the attempted purification of trypanosomal α-GPDH, various SDS-gels were run and stained for protein either with Coomassie Blue or by silver-staining, in order to visualise the effect of some of the more promising techniques.

4.12: Non-specific adsorption

The adsorbants investigated were Alumina Cγ (Willstätter's Cγ modification of aluminium hydroxide gel), calcium phosphate gel Type I (Aged), calcium phosphate gel Type II Neutral (Brushite) and hydroxyapatite. The percentage solid in the supplied gels was determined by drying to a constant mass and once known, these values were used to prepare suspensions of the gels in 5mM phosphate buffer, pH 6.5, to a 1:1 ratio of gel solid to protein content of the sample to be adsorbed. An extremely brief period of sonication was carried out (a maximum of five seconds) to aid dispersal of the suspension.

Fractional adsorption was then carried out on 14.5KP(T) extracts with each adsorbant as described by Colowick (1955). The period of incubation was 15 minutes, on ice, with frequent agitation, the mixture then being centrifuged at 1000 x g for three minutes. The supernatant was assayed for protein concentration and α-GPDH activity. The adsorption pattern for each of the four gels used is shown as Figure 26. The technique was consequently pursued only with respect to Alumina Cγ at a gel solid:protein ratio of 1:1.

The pellet of Alumina Cγ with adsorbed enzyme thus obtained was then washed once with 5mM phosphate buffer, pH 6.5. The washed pellet was then subjected to stepwise elutions of α-GPDH activity. The

Figure 26: Non-specific adsorption of trypanosomal α-GPDH by

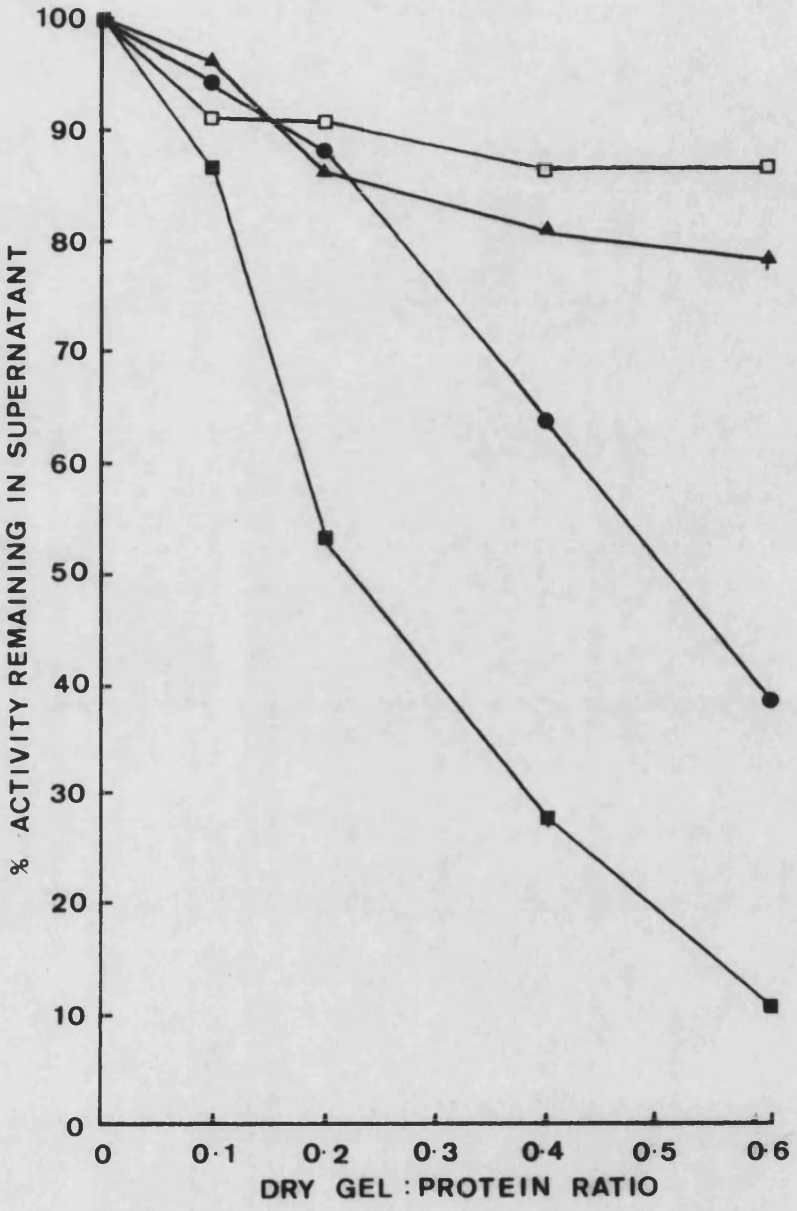
□ brushite

▲ hydroxyapatite

● calcium phosphate gel type 1

■ alumina Cγ

The percentage solid of each gel was determined and a suspension of each prepared in 5mM phosphate buffer, pH 6.5, to a 1:1 ratio of gel solid to protein content of the sample to be adsorbed. Brief sonication aided dispersal of the suspensions. Gel suspensions and 14.5KP(T) samples were then mixed to yield dry gel:sample protein ratios of 0.1 to 0.6 (ie. 0.2ml gel suspension mixed with 1ml enzyme extract yielded a ratio of 0.2). Each mixture was incubated for 15 minutes on ice, followed by centrifugation at 1000 x g for three minutes. The supernatant was then assayed for protein content and enzyme activity.


100
90
80
70
60
50
40
30
20
10
0
% ACTIVITY REMAINING IN SUPERNATANT
0
0·1
0·2
0·3
0·4
0·5
0·6
DRY GEL : PROTEIN RATIO

eluant was 0 to 0.5M phosphate buffer, pH 8.0, and the mixture was incubated with agitation for 15 minutes on ice and then spun as above. The supernatant was removed for assay and the procedure repeated. The fractions showing a significant increase in specific activity were pooled for further purification. A typical set of results is shown in Table 19.

The recovery of activity in this case was 71% and the increase in specific activity was 3.4-fold. Non-specific adsorption using Alumina Cγ proved to be an extremely reliable method and was consequently included in the purification scheme for trypanosomal α-GPDH.

4.13: Hydrophobic-interaction chromatography

Hydrophobic-interaction chromatography with phenyl-sepharose has been used successfully by Misset and Opperdoes (1984) for the separation of a mixture of nine trypanosomal glycosomal enzymes. During their work no enzyme was eluted alone and further purification was required in each case; α-GPDH co-eluted with phosphofructokinase at between 30 and 35% (v/v) on a 0 to 70% (v/v) linear gradient of ethylene glycol. For this work the only enzyme of interest was α-GPDH and to this end the method of Misset and Opperdoes (1984) was necessarily substantially altered.

Phenyl-sepharose resin was equilibrated with 25mM Tris-HCl buffer containing 5mM EDTA, final pH 7.8. After being packed into a column of suitable dimensions the phenyl-sepharose was further equilibrated with Tris-HCl buffer which contained 5mM EDTA, 1mM dithiothreitol (DTT), 1μM leupeptin and 50mM ammonium sulphate, final pH 7.8

Table 19: The adsorption to and elution from Alumina Cγ of α-GPDH from T. brucei

Sample	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	% recovery	Protein conc. (mg/ml)	Specific activity (U/mg)
14.5KP(T)	14.0	5.38	75.32	100	3.350	1.60
S'natant	19.5	0.19	3.70	4.91	0.239	0.79
Wash	7.0	0.01	0.07	0.09	0.053	0.19
Eluate:						
50mM	10.0	0.04	0.40	0.53	0.146	0.27
100mM	10.0	0.24	2.40	3.19	0.256	0.94
150mM	10.0	2.26	22.60	30.00	0.347	6.51
200mM	10.0	2.47	24.70	32.79	0.278	8.88
250mM	10.0	1.33	13.30	17.66	0.187	7.11
300mM	10.0	0.92	9.20	12.2	0.179	5.14
500mM	10.0	0.46	4.60	6.1	0.260	1.77
Combined 150, 200, 250mM eluates	28.0	1.91	53.48	71.00	0.347	5.50

(chromatography buffer). Enzyme extract was applied to the column and washed through with several column volumes of chromatography buffer. Fractions of the eluant obtained during loading and the subsequent wash were assayed for α-GPDH activity. A linear gradient of 0-85% (v/v) ethylene glycol in chromatography buffer was applied to the column at a rate of up to 16ml/hour and fractions collected at a suitable rate. This method was included in the final purification scheme when the precise method used was as described below.

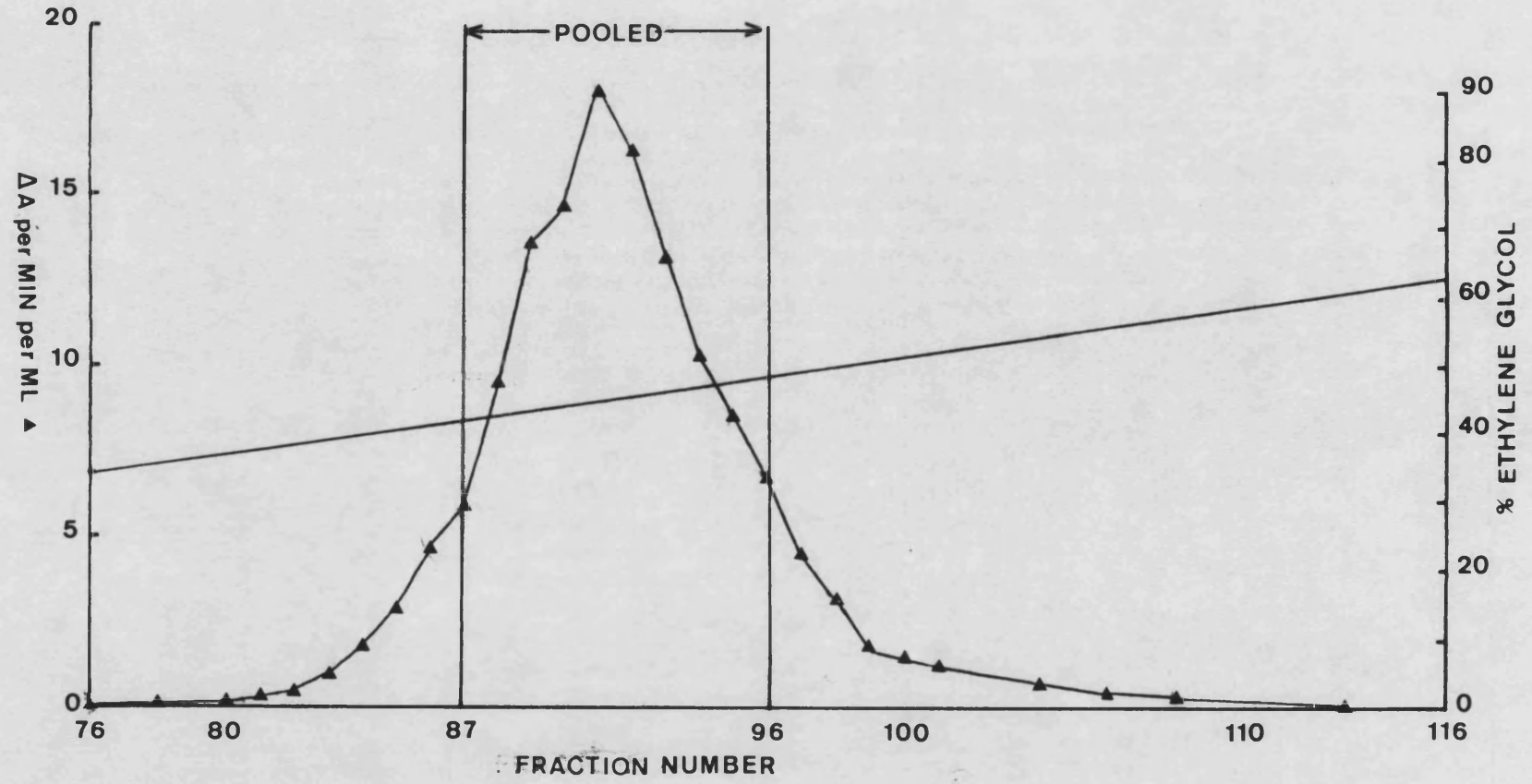
Equilibrated phenyl-sepharose was packed into an 18.0 x 1.6cm column and washed with several column volumes of chromatography buffer (see above). The appropriate enzyme extract, up to 30ml in volume, was applied to the column at 35ml/hour by means of a peristaltic pump. The loaded column was then washed with chromatography buffer. During loading and washing, fractions were collected and assayed for α-GPDH activity; no leakage of enzyme was found during any run of the column. A 0-85% (v/v) linear gradient of ethylene glycol in chromatography buffer, total volume 200ml, was then applied to the column at 15ml/hour and fractions collected every six minutes. The recovery of α-GPDH activity consistently occurred between 35 and 60% (v/v) ethylene glycol as determined by refractive index measurements, a typical peak being shown in Figure 27.

The protein content of the fractions proved difficult to measure, the presence of ethylene glycol interfering with the Bio-Rad dye-binding method, absorbance at 260/280nm and the Lowry method (Bensadoun and Weinstein, 1976; Peterson, 1979). This problem was overcome by Misset and Opperdoes (1984) by the use of the fluorescamine method (Bohlen et al., 1973) but for the work reported

here an approximation of the protein content of the pooled peak fractions was obtained by the modified Lowry method of Pace et al. (1974). After use, the column was regenerated by washing with one column volume of distilled water followed by column volumes of ethanol, butan-1-ol, ethanol and finally a further volume of distilled water. The resin was then re-equilibrated as described above.

The typical overall recovery of α-GPDH activity from a phenyl-sepharose chromatography column was over 100%, that from the peak in Figure 27 being 103.3%. The most active fractions were pooled, yielding a recovery of 80.9% activity. The protein content of this pool was estimated as approximately 12.5% of that applied, leading to an obviously significant increase in specific activity although one which could not be reliably quantified. The pooled fractions were retained for use in the next stage of the purification scheme.

Figure 27: The recovery of trypanosomal α-GPDH from a 18.0 x 1.6 cm column of phenyl sepharose equilibrated with 25mM Tris-HCl buffer containing 5mM EDTA, 1mM DTT, 1μM leupeptin and 50mM ammonium sulphate, pH 7.8. Up to 30ml enzyme extract was loaded onto the column at 35ml/hr and the column washed through with one volume of the buffer described above. Elution of the column was then carried out with a 200ml 0 - 85% (w/v) linear gradient of ethylene glycol in the above buffer, applied to the column at 15ml/hr. Fractions were collected every six minutes. The typical recovery of α-GPDH for such a column was over 100%, the most active fractions being pooled to yield approximately 80.9% of applied activity with an estimated increase in specific activity of approximately 3-fold.


POOLED
20
15
10
5
0
ΔA per MIN per ML
90
80
60
40
20
0
% ETHYLENE GLYCOL
76
80
87
96
100
110
116
FRACTION NUMBER

4.14: The purification scheme

The purification scheme finally used involved a combination of the most useful and reliable of the methods previously described. A 14.5KP(T) extract was prepared with leupeptin and antipain included in the disruption buffer at 10 and 20μg/ml respectively. The 14.5KP(T) extract was then subjected to adsorption and elution from Alumina Cγ and the most active eluates combined before being applied to a phenyl-sepharose hydrophobic interaction chromatography column. The peak fractions eluted from the column were pooled and this pool then adsorbed once again to Alumina Cγ and eluted exactly as for the first adsorption. The fractions were not pooled however but the protein concentration of each was estimated. A sample of the fraction with the highest specific activity was applied to an SDS-polyacrylamide gel with samples from each of the other stages and molecular weight markers. Having determined the electrophoretic purity of this final fraction and established the absence of traces of contaminating enzymes, particularly triose phosphate isomerase, the fraction had leupeptin and antipain added to it as before.

This fraction was then used for investigation of the kinetic behaviour of trypanosomal NAD^+-linked α-GPDH. A typical purification table is shown as Figure 28. Although the final yield of α-GPDH activity was low using this purification scheme, the activity and stability of the enzyme was sufficient for kinetic studies to be carried out.

Figure 28: Typical values obtained during the purification of α-GPDH from T. brucei by the method described in Section 4.14

Sample	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (-fold)	Yield (%)
Homogenate	25.50	91.29	149.180	0.612	1	100
14.5KP(T)	10.20	90.04	21.160	4.255	6.95	98.6
Alumina Cγ fraction 1	15.87	57.05	3.396	16.799	27.45	62.5
Phenyl-sepharose eluates	25.39	46.16	NA	NA	NC	50.6
Alumina Cγ fraction 2	17.77	4.49	0.213	21.080	34.44	4.9

CHAPTER 5: THE KINETIC BEHAVIOUR OF TRYPANOSOMAL $\bar{\alpha}$-GPDH

5.1: The methods used

5.1.1: The accurate determination of NADH and DHAP concentrations

In order that the exact required substrate concentrations should be used for kinetic measurements, approximate stock solutions were prepared in assay buffer (50mM phosphate buffer plus 8mM $MgCl_2$, pH 7.4) and then estimated as described below. The concentration of NADH was determined by dilution of a sample of the stock solution with assay buffer and measurement of the absorbance at 340nm versus a buffer blank. The molar absorption coefficient for NADH is 6.22×10^3 /litre/cm/mole (ie a 1mM solution of NADH has an absorbance of 6.22 units at 340nm) and from this value the concentration of any solution of NADH was calculated.

The concentration of a solution of DHAP was determined enzymatically. Into a 1ml plastic cuvette was placed:

5mM NADH	100µl
assay buffer	840µl
Enzyme extract	50µl

When an absorbance base-line had been established 10µl DHAP solution was added and the decrease in absorbance recorded until no further change was seen. The enzyme extract used was a 14.5KP(T) sample of sufficient activity to catalyse the reaction occuring, which was:

$$NADH + H^+ + DHAP \xrightarrow{\alpha\text{-GPDH}} \alpha\text{-GP} + NAD^+$$

In the presence of an excess of NADH, the change in absorbance was proportional to the concentration of DHAP in the assay. Since DHAP and NADH react in equimolar quantities the change in absorbance was related to DHAP concentration by calculation using the molar absorption coefficient of NADH as before.

5.1.2: Kinetic measurements

The reaction was followed as before by measurement of the rate of disappearance of NADH at 340nm and 37°C. Kinetic experiments were carried out in plastic cuvettes with a 1ml volume reaction mixture. All components of the reaction were prepared in 50mM phosphate buffer with 8mM $MgCl_2$, pH 7.4 (assay buffer) and the pH of each solution adjusted to 7.4. The solutions were all kept on ice with the exception of the buffer itself which was incubated at 40.3°C. The cuvette-holder of the Cecil CE 212 spectrophotometer used had a water-jacket which was found to be maintained at 37°C when the circulating water was at 40.3°C.

All reactants but the enzyme were placed into the cuvette and left to stand in the cuvette-holder for a minimum of seven minutes before the reaction was started, this having been determined as sufficient time for the reaction mixture to reach 37°C. The enzyme extract, 10µl in volume, was added to initiate the reaction. The chart speed and scale expansion were used to ensure that the progress

of each reaction was linear and simple to determine from the initial rate. Each assay was carried out in duplicate and a "standard assay" run with each experiment; this standard assay, containing 0.2mM NADH and 0.5mM DHAP as substrates, was run in triplicate with 10µl of the pure enzyme solution and the rates obtained used to adjust the experimental values to a common starting point. The investigations carried out were as follows:

A) To investigate any inhibitory effect on α-GPDH activity of α-GP at 0, 0.03, 0.06 and 0.1M.

B) To investigate any inhibitory effect on α-GPDH activity of NAD^+ at 0, 0.5 and 5.0mM.

C) Control experiments to determine whether α-GPDH activity was affected by 0.1M phosphate buffer and 0.1M β-GP.

The concentrations quoted above were all final concentrations in the cuvette, α-GP being added at 0.5M, NAD^+ at 50mM, β-GP at 0.1M and phosphate buffer at 0.25M. Each experiment was carried out using a grid system as shown in Figure 29, stock solutions of NADH and DHAP being 0.5mM and 10mM respectively and effectors added when required. The volume of assay buffer was adjusted to maintain the total assay volume at 1ml.

Figure 29: The grid system used during the kinetic studies of α-GPDH from T. brucei

NADH / DHAP	·005	·01	·025	·05
·20				
·75				
1·5				
5·0				

5.2: Results obtained from the kinetic study of trypanosomal α-GPDH

5.2.1: Determination of kinetic parameters in the absence of inhibitors

Kinetic data were obtained by measuring reaction rates spectrophotometrically at each of four concentrations of DHAP and NADH (see section 5.1.2); thus a 4 x 4 grid of values was obtained. All kinetic measurements were made in duplicate.

The primary data were analysed by the direct linear plot (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1978) in blocks, firstly treating DHAP as the variable substrate at each of the four fixed NADH concentrations. From this analysis estimates of $V_{max(app)}$ and $V_{max(app)}/K_{m(app)}$ were obtained. These were then treated as the variables in secondary plots against the fixed (NADH) values to give estimates of the four kinetic parameters V_{max}, $K_{m(DHAP)}$, $K_{m(NADH)}$ and K_{ab} according to the equation:

$$v = \frac{Vmax}{1 + \frac{K_{m(NADH)}}{(NADH)} + \frac{K_{m(DHAP)}}{(DHAP)} + \frac{K_{ab}}{(NADH)(DHAP)}}$$

Standard errors were calculated from 68% confidence limits given by

the analysis.

The analysis was then repeated in a converse manner, treating NADH as the variable and DHAP as the fixed substrate. The resulting estimates of the kinetic constants were weighted according to the reciprocal of the variance and the results given below represent weighted means:

Table 20 : Kinetic constants for trypanosomal α-GPDH

Kinetic constant	Value
V_{max}	64.9μmol/min/mg protein
K_{ma}	0.125 ± 0.043mM
K_{mb}	13.6 ± 4.7mM
K_{ab}	0.076 ± 0.008mM^2

where a is NADH and b is DHAP. The calculation of V_{max} is shown in Figure 30.

Figure 30: The calculation of V_{max} for the α-GPDH of T. brucei

17.77mls extract contained 0.213mg pure enzyme (see Figure 28).

Therefore 10μl enzyme extract contained 1.199×10^{-4}mg pure enzyme.

Vmax was 26.69μmol NADH/min/0.012mg.

Therefore Vmax was 2.224mmol NADH/min/mg pure enzyme.

0.213mg was 4.9% original activity.

Therefore 4.347mg α-GPDH/2.55×10^{10} cells, equivalent to 9.668mmol/min/2.55×10^{10} cells.

Vmax = 0.0379mmol/min/1×10^{8}cells.

During this work 1×10^{8} cells was estimated as containing 0.584mg protein.

So Vmax = 0.0379mmol/min/0.584mg protein, equivalent to 0.0649mmol/min/mg protein.

5.2.2: Determination of the effect of product inhibition on the kinetic behaviour of trypanosomal α-GPDH and prediction of a possible kinetic mechanism

Examples of graphical representation of the kinetic data obtained from assays carried out in the absence of inhibitors and in the presence of NAD^+ and α-GP as inhibitors are shown as Figures 31 and 32, 33 to 36 and 37 to 40 respectively. The parameters obtained are tabulated in Figures 41, 42, 43 and 44. From these results certain trends were apparent and these are summarised in Table 21.

The following conclusions can thus be drawn:

a) Using NAD^+ as an inhibitor with varying NADH and fixed DHAP, the $V_{max(app)}$ value remained approximately constant while the $K_{m(app)}/V_{max(app)}$ value increased with increasing concentration of NAD^+, showing that NAD^+ is a competitive inhibitor with respect to NADH.

b) When NAD^+ was acting as an inhibitor at varying concentrations of DHAP and fixed NADH, the value of $V_{max(app)}$ decreased while the value of $K_{m(app)}/V_{max(app)}$ increased with increasing concentration of NAD^+, indicative of mixed inhibition.

c) When α-GP was acting as an inhibitor with respect to NADH, the value of $V_{max(app)}$ decreased while the value of $K_{m(app)}/V_{max(app)}$ increased with increasing concentration of α-GP, showing mixed inhibition.

d) With α-GP acting as an inhibitor at varying DHAP and fixed NADH, the value of $V_{max(app)}$ decreased while the value of $K_{m(app)}/V_{max(app)}$ increased with increasing concentration of α-GP, again showing mixed inhibition.

Inhibitor constants (K_i values) were determined from the graphs shown as Figures 45 to 48. The lines drawn on these graphs were calculated as lines of linear regression using weighted $K_{m(app)}/V_{max(app)}$ values, where the weighting was proportional to the reciprocal of the standard error for that value. The values obtained are shown in Table 22.

From the results obtained it was possible to deduce a probable kinetic mechanism for trypanosomal α-GPDH. The S/V versus S plots in Figures 31 and 32 both clearly show that the lines drawn intersect to the left of the y-axis, therefore eliminating the ping-pong (substituted enzyme) mechanism in which the lines should intersect on the y-axis. The general uninhibited rate equation thus implied was:

$$v = \frac{V_{max}}{1 + \frac{K_{ma}}{(A)} + \frac{K_{mb}}{(B)} + \frac{(K_{sa})(K_{mb})}{(A)(B)}}$$

The three most common kinetic mechanisms consistent with this equation are the rapid random equilibrium mechanism, the compulsory-order mechanism and the Theorell-Chance mechanism. The three may be distinguished by their different patterns of product inhibition. Where the reaction occurring is $A + B \longrightarrow P + Q$ (assuming A binding to the enzyme first and P being released from the enzyme last for both the compulsory order and Theorell-Chance mechanisms) then the patterns of product inhibition are as shown in Table 23.
The results obtained for trypanosomal α-GPDH were therefore only consistent with a compulsory-order kinetic mechanism with NADH (represented by A in Table 23) binding first and NAD^+ (represented by B) being released last.

Having concluded that a compulsory-order mechanism was in operation, it was apparent that the K_{ab} value obtained in Section 5.2.1 was equivalent to $K_{sa}K_{mb}$ (see above). The K_s value for NADH was thus calculated as 5.588μM.

Table 21: Variation of values of kinetic parameters with increasing inhibitor concentration

Inhibitor	Varied substrate	Fixed substrate	$V_{max(app)}$	$K_{m(app)}/V_{max(app)}$
NAD^+	NADH	DHAP	constant	increase
NAD^+	DHAP	NADH	decrease	increase
α-GP	NADH	DHAP	decrease	increase
α-GP	DHAP	NADH	decrease	increase

Table 22: Inhibitor constants for trypanosomal α-GPDH

Inhibitor	Acting upon	K_i (mM)
NAD^+	DHAP	infinity
NAD^+	NADH	4.122 ± 0.052
α-GP	DHAP	12.3 ± 0.8
α-GP	NADH	55.8 ± 3.9

Table 23: Product inhibition patterns for three bisubstrate mechanisms where the concentration of the fixed substrate is not saturating (Comp. = competitive)

Mechanism	Variable substrate	Product acting as inhibitor	
		P	Q
Rapid random equilibrium	A	Comp.	Comp.
	B	Comp.	Comp.
Compulsory order	A	Comp.	Mixed
	B	Mixed	Mixed
Theorell-Chance	A	Comp.	Mixed
	B	Mixed	Comp.

Figure 31: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the absence of inhibitors. The substrate represented by S on the graph was NADH and the concentrations of DHAP were as follows:

○ 0.20mM DHAP

● 0.75mM DHAP

□ 1.50mM DHAP

■ 5.00mM DHAP

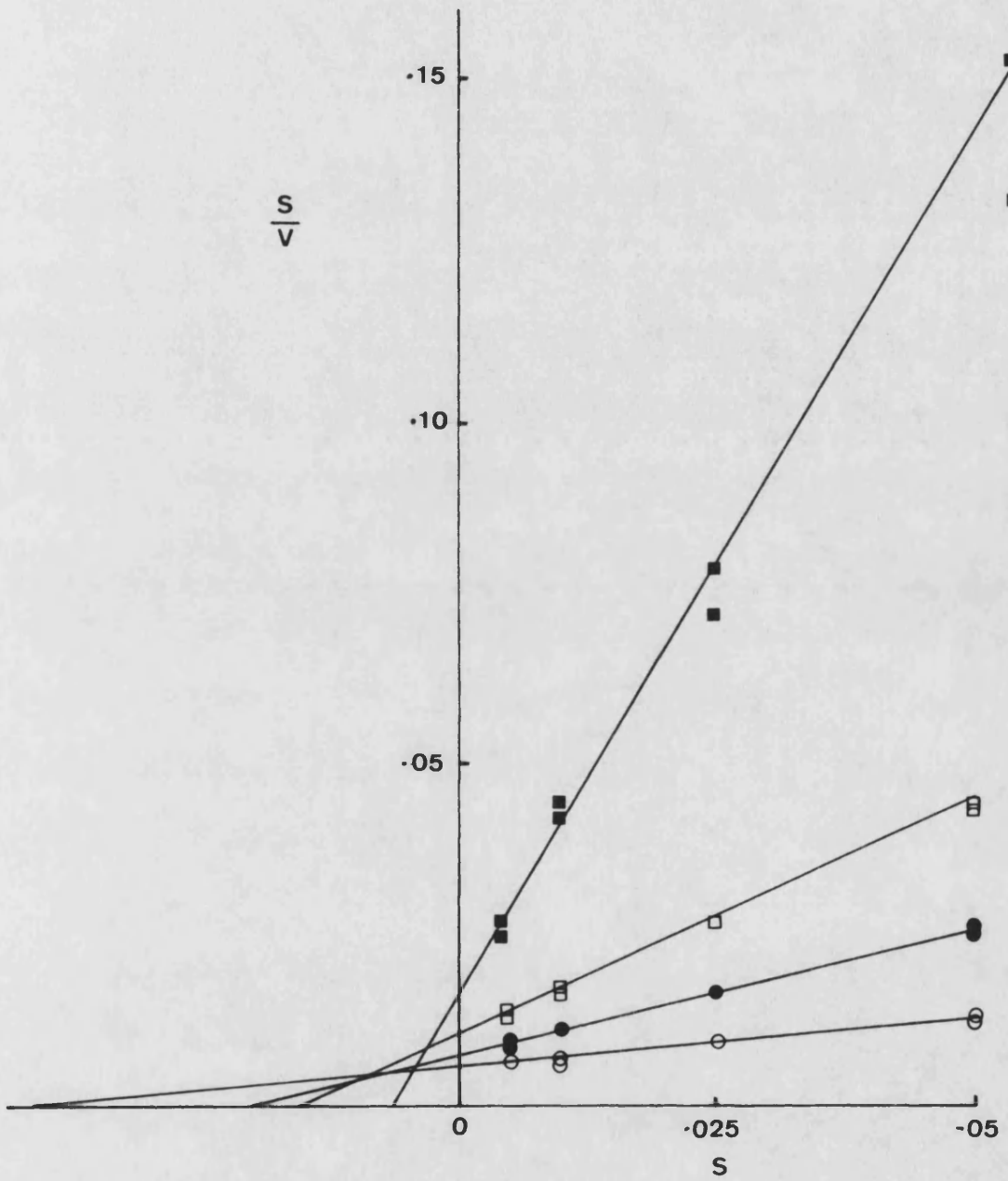

·15
·10
·05
S/V
0
·025
·05
S

Figure 32: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the absence of inhibitors. The substrate (S) is DHAP and the concentrations of NADH were as follows:

○ 0.005mM NADH

● 0.010mM NADH

□ 0.025mM NADH

■ 0.050mM NADH

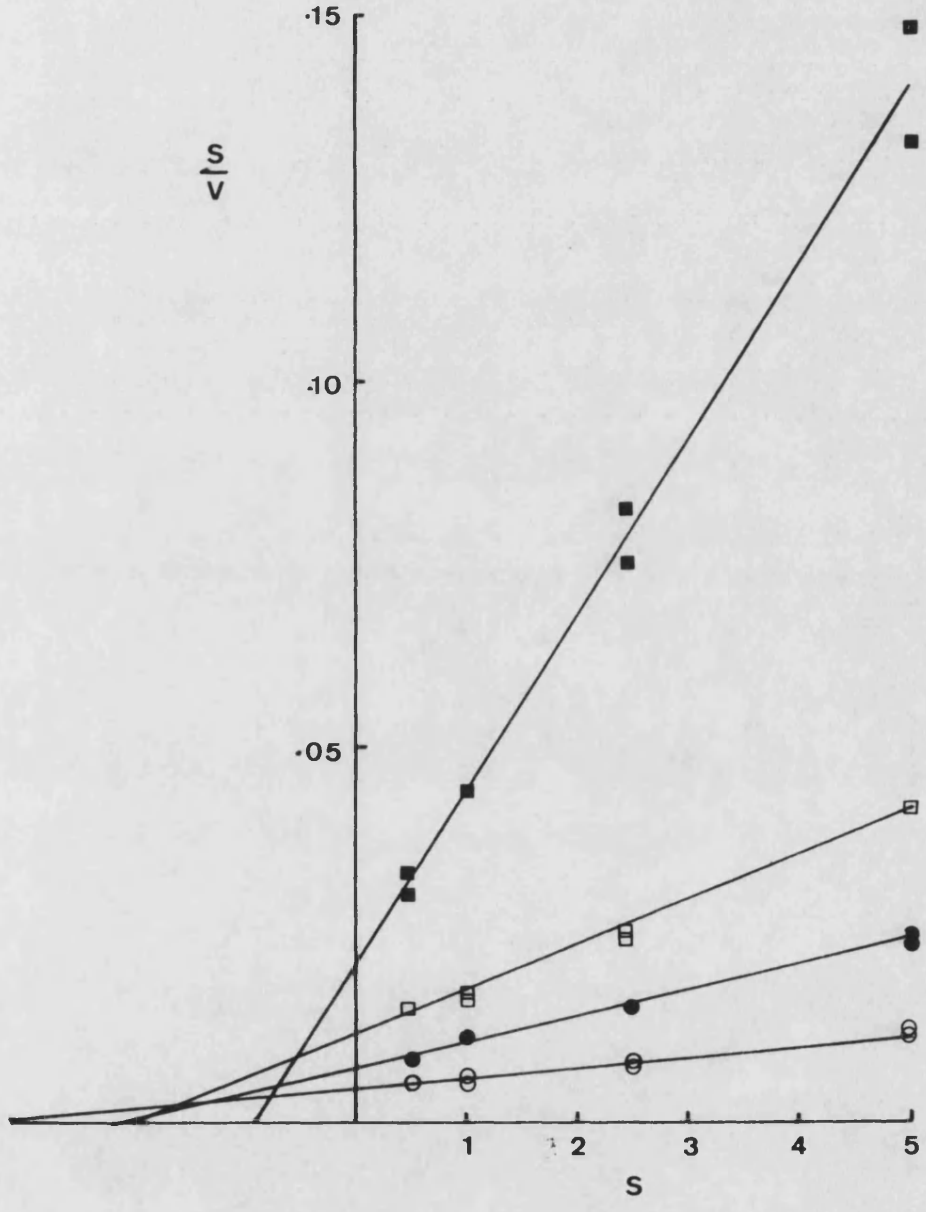

·15
·10
·05
S/V
1
2
3
4
5
S

Figure 33: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of NAD^+ as inhibitor. The variable substrate (S) was DHAP and the concentration of NADH was fixed at 0.025mM. The concentrations of inhibitor were as follows:

0mM NAD^+ □

0.5mM NAD^+ ▲

5.0mM NAD^+ △

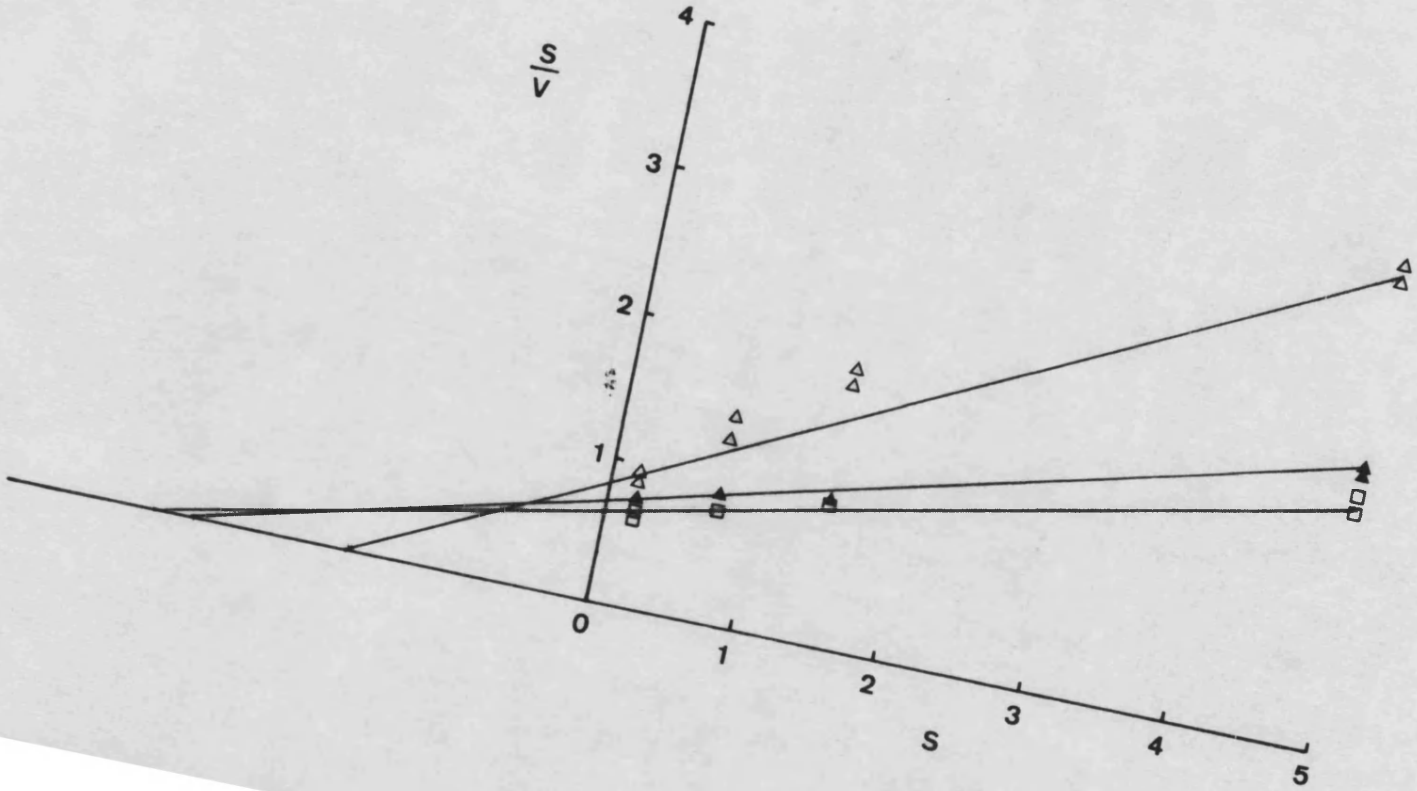

S/V
4
3
2
1
0
1
2
3
4
5
S

Figure 34: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of NAD^+ as inhibitor. The variable substrate (S) was DHAP and the concentration of NADH was fixed at 0.05mM. The concentrations of inhibitor were as follows:

0mM NAD^+ □

0.5mM NAD^+ ▲

5.0mM NAD^+ △

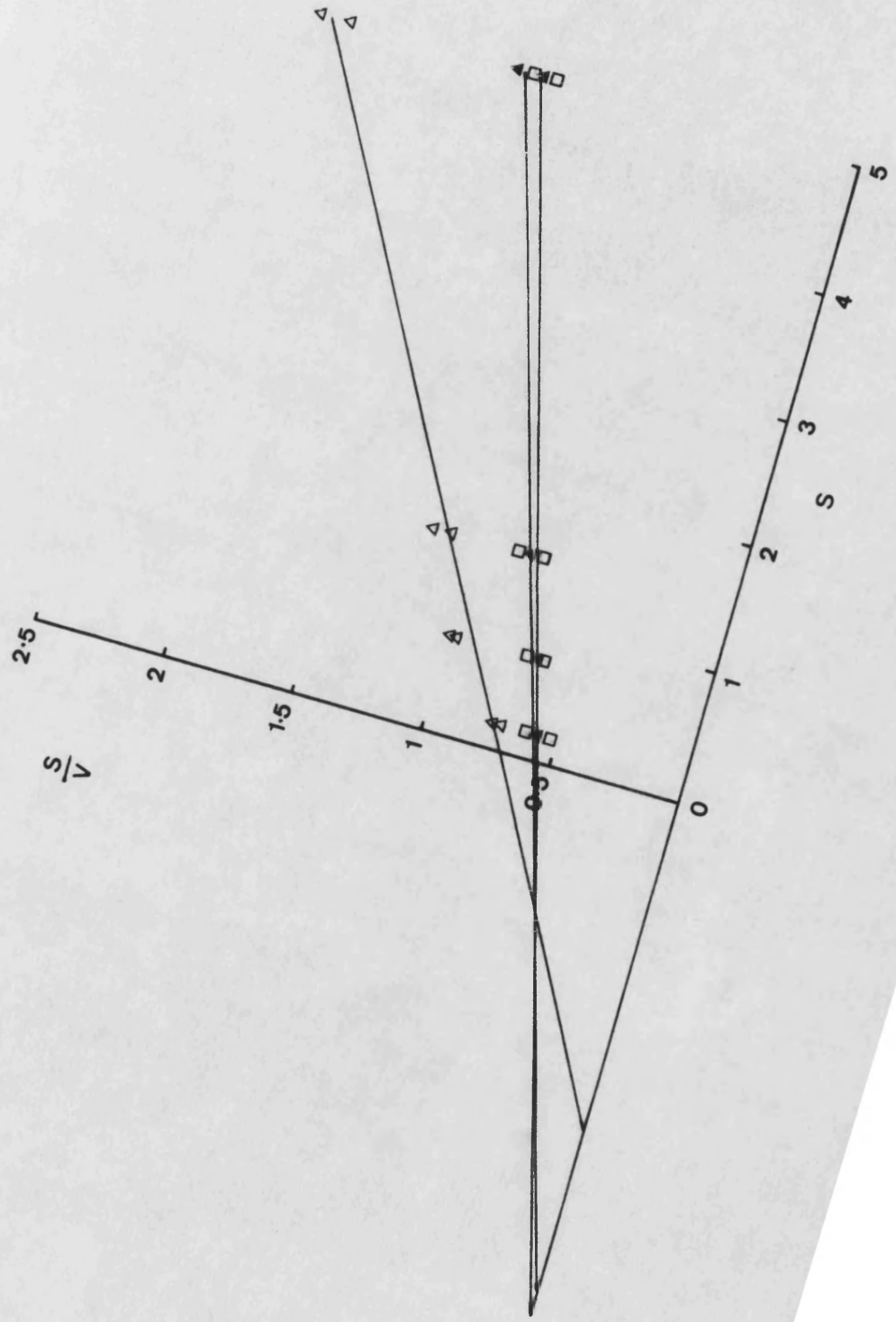

S/V
2·5
2
1·5
1
0·5
0
1
2
3
4
5
S

Figure 35: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of NAD^+ as inhibitor. The variable substrate (S) was NADH and the concentration of DHAP was fixed at 1.5mM. The concentrations of inhibitor were as follows:

0mM NAD^+ □

0.5mM NAD^+ ▲

5.0mM NAD^+ △

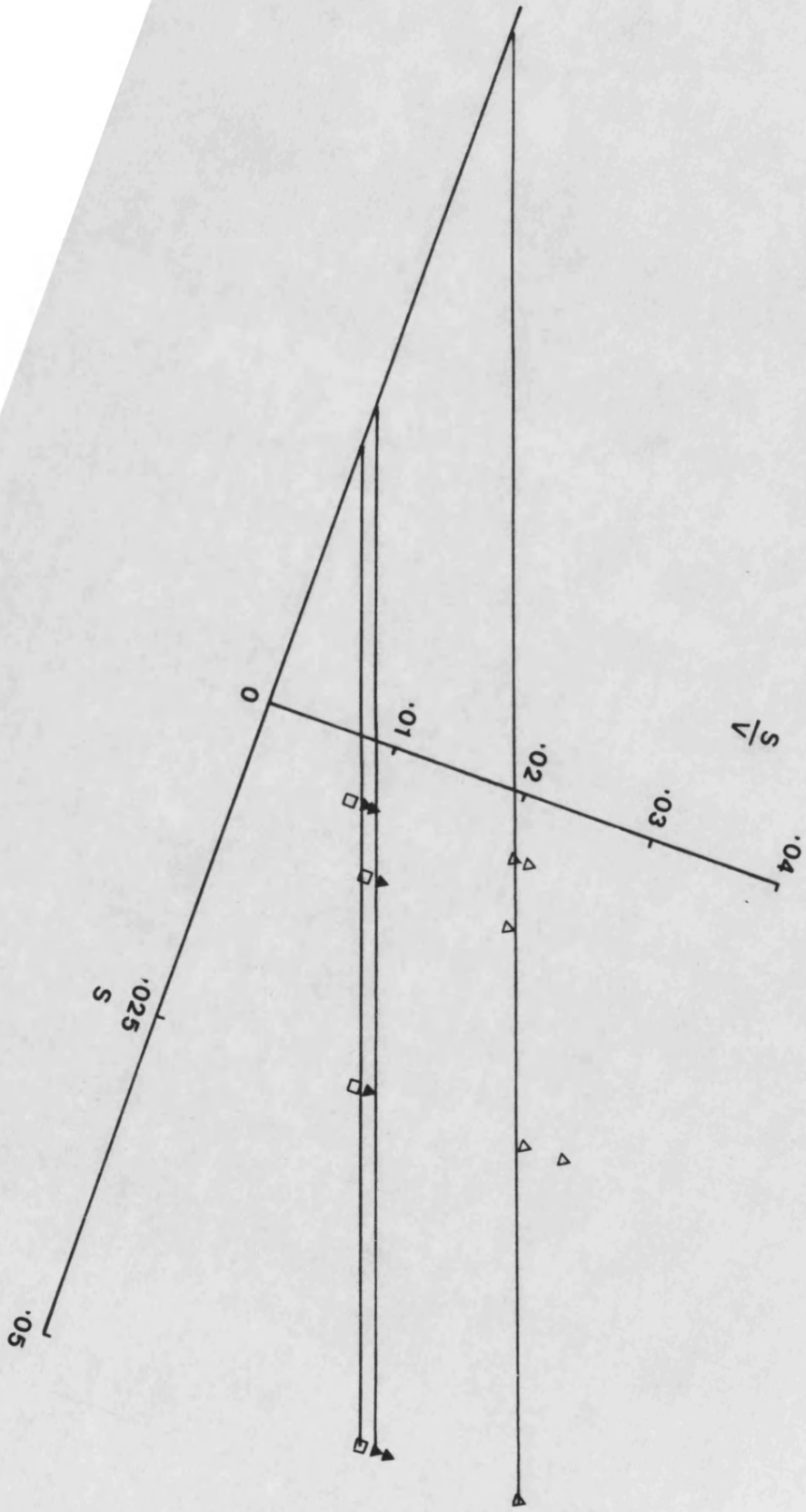

0
.01
.02
.03
.04
s/v
.025
s
.05

Figure 36: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of NAD^+ as inhibitor. The variable substrate (S) was NADH and the concentration of DHAP was fixed at 5.0mM. The concentrations of inhibitor were as follows:

0mM NAD^+ □

0.5mM NAD^+ ▲

5.0mM NAD^+ △

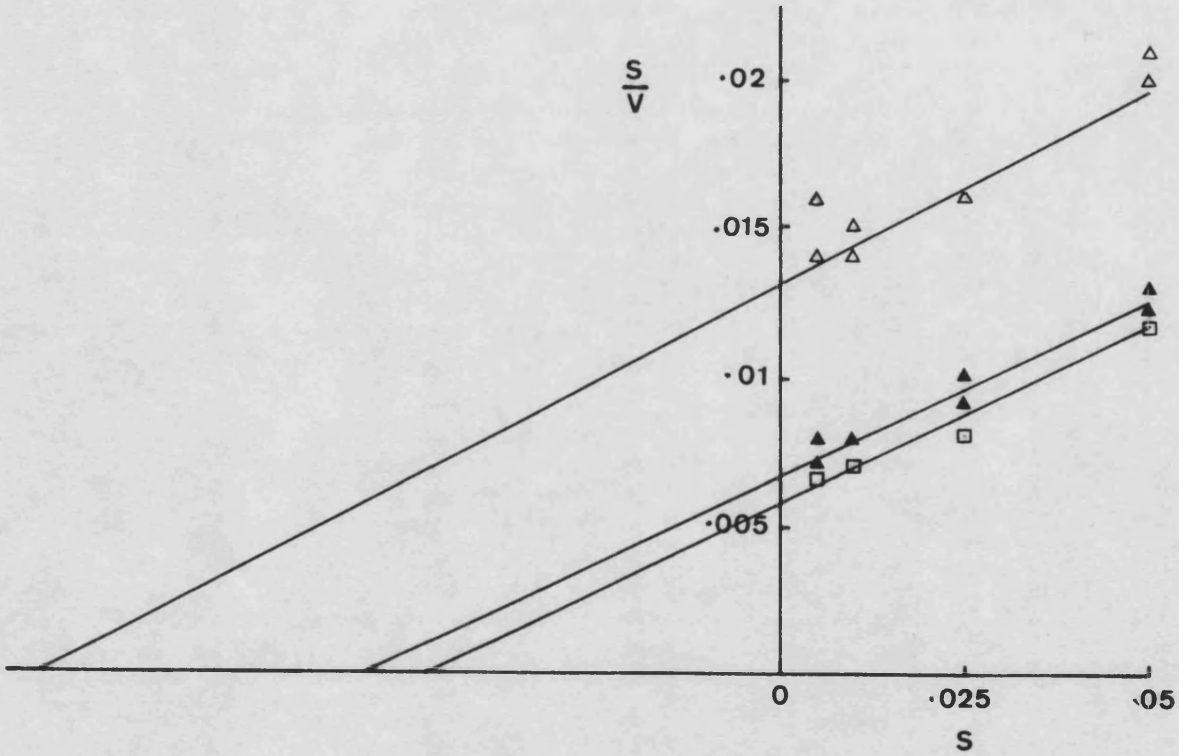

S/V
·02
·015
·01
·005
0
·025
·05
S

Figure 37: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of α-GP as inhibitor. The variable substrate (S) was DHAP and the concentration of NADH was fixed at 0.025mM. The concentrations of inhibitor were as follows:

● 0.00M α-GP

○ 0.03M α-GP

■ 0.06M α-GP

□ 0.10M α-GP

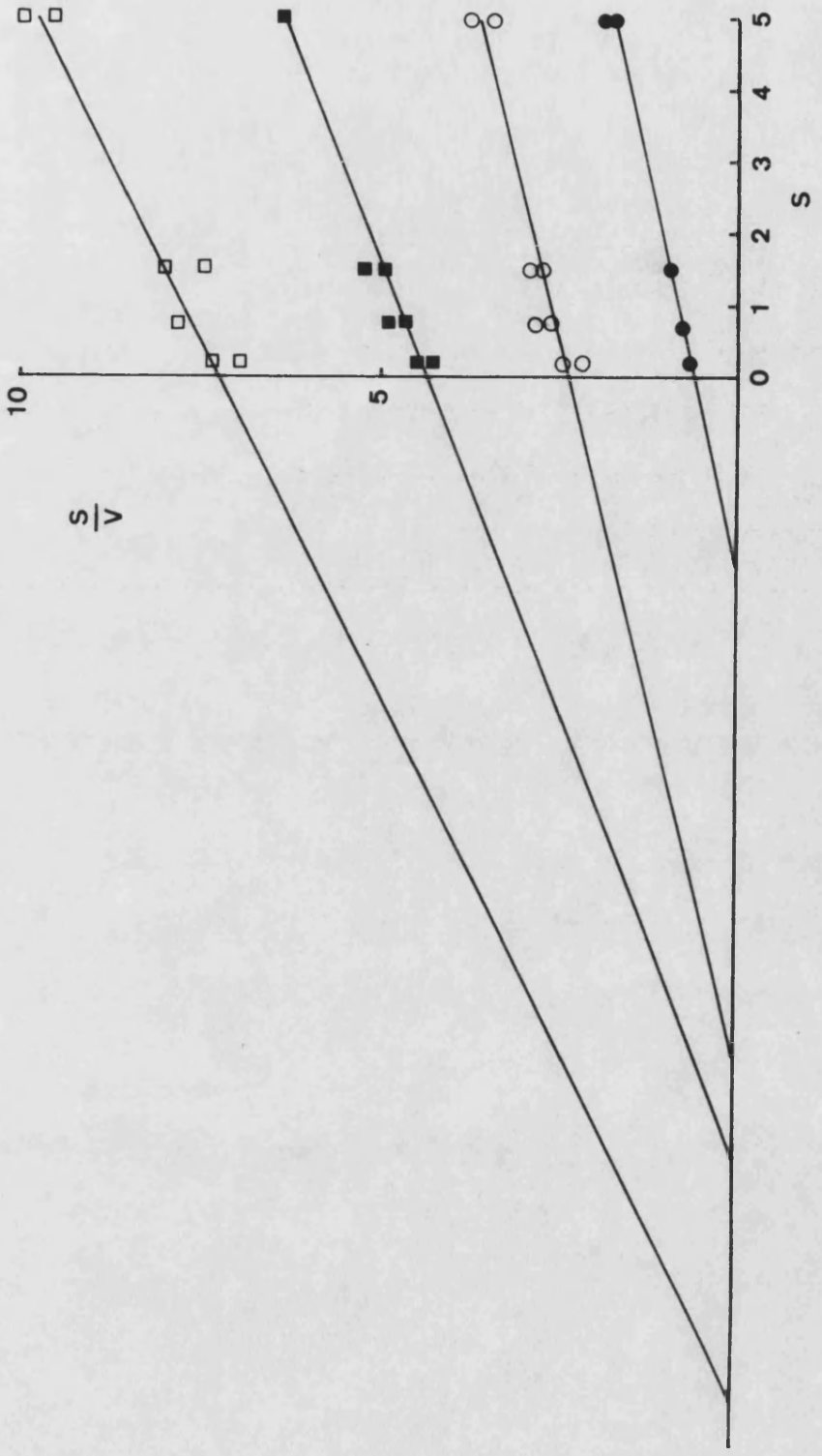

10
5
S/V
0
1
2
3
4
5
S

Figure 38: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of α-GP as inhibitor. The variable substrate (S) was DHAP and the concentration of NADH was fixed at 0.01mM. The concentrations of inhibitor were as follows:

● 0.00M α-GP

○ 0.03M α-GP

■ 0.06M α-GP

□ 0.10M α-GP

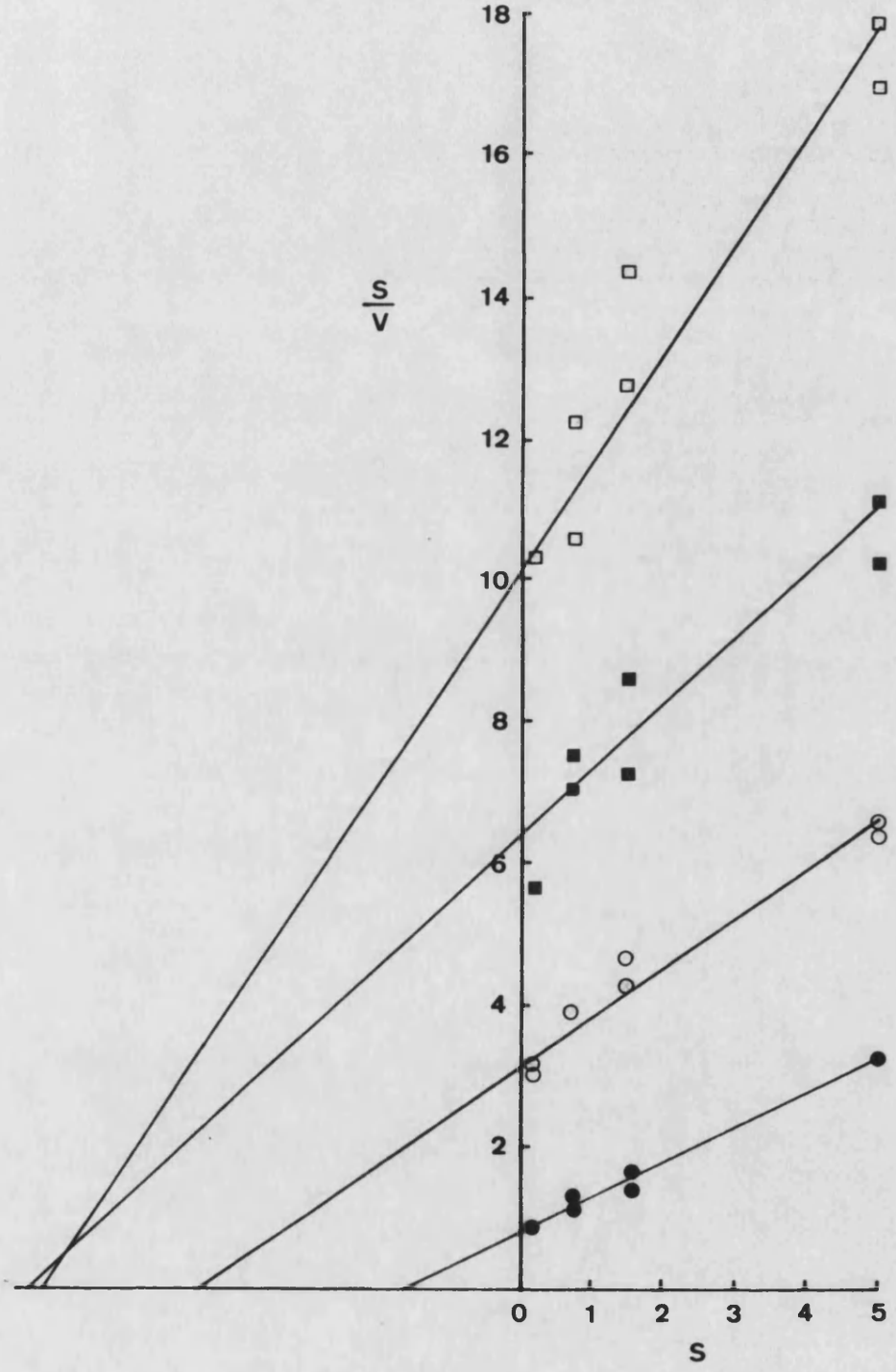

18
16
14
12
10
8
6
4
2
S/V
0
1
2
3
4
5
S

Figure 39: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of α-GP as inhibitor. The variable substrate (S) was NADH and the concentration of DHAP was fixed at 0.75mM. The concentrations of inhibitor were as follows:

● 0.00M α-GP

○ 0.03M α-GP

■ 0.06M α-GP

□ 0.10M α-GP

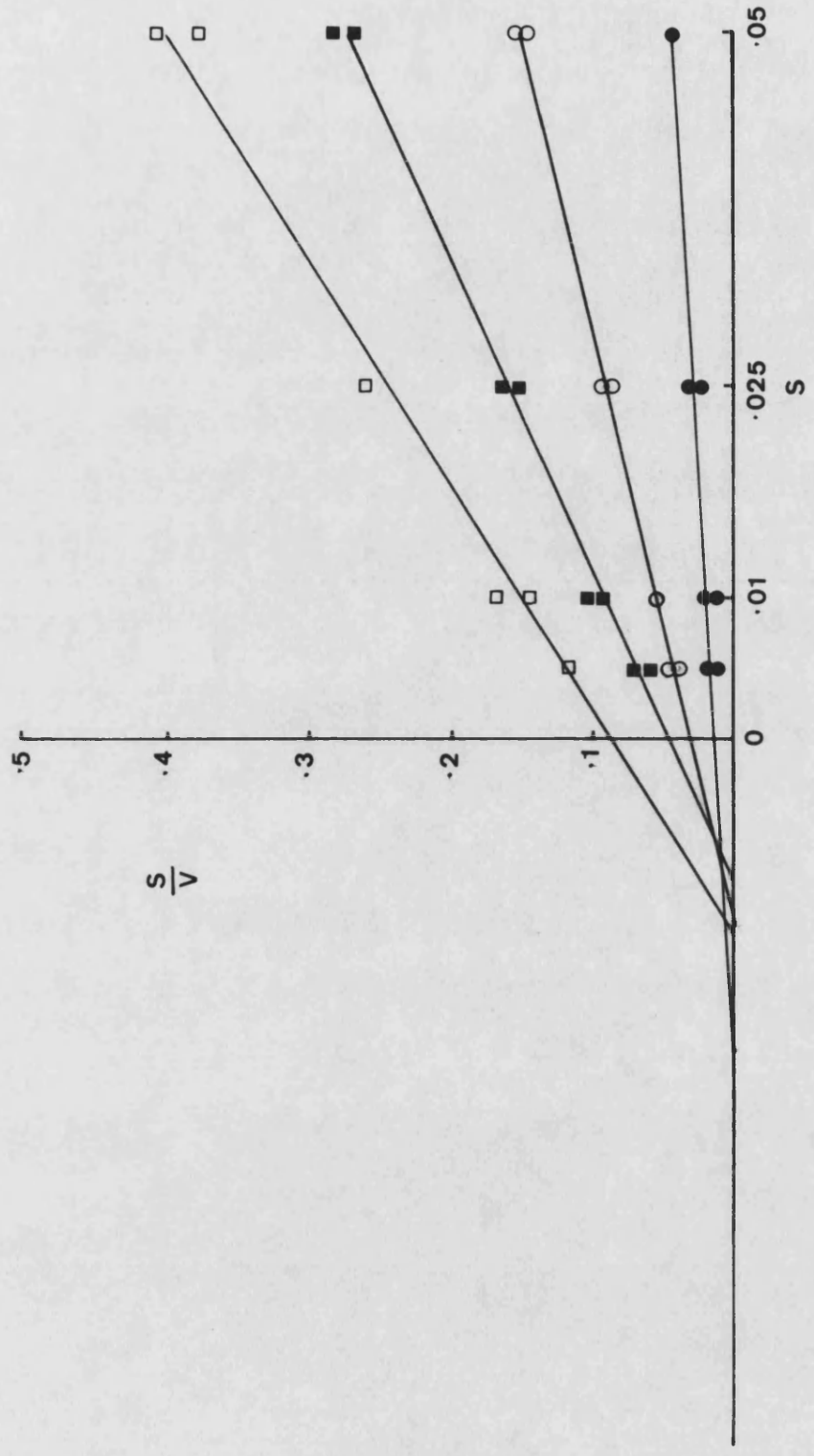

S/V
·5
·4
·3
·2
·1
0
·01
·025
·05
S

Figure 40: Half-reciprocal primary plot (Hanes plot) for the activity of α-GPDH in the presence of α-GP as inhibitor. The variable substrate (S) was NADH and the concentration of DHAP was fixed at 5.0mM. The concentrations of inhibitor were as follows:

● 0.00M α-GP

○ 0.03M α-GP

■ 0.06M α-GP

□ 0.10M α-GP

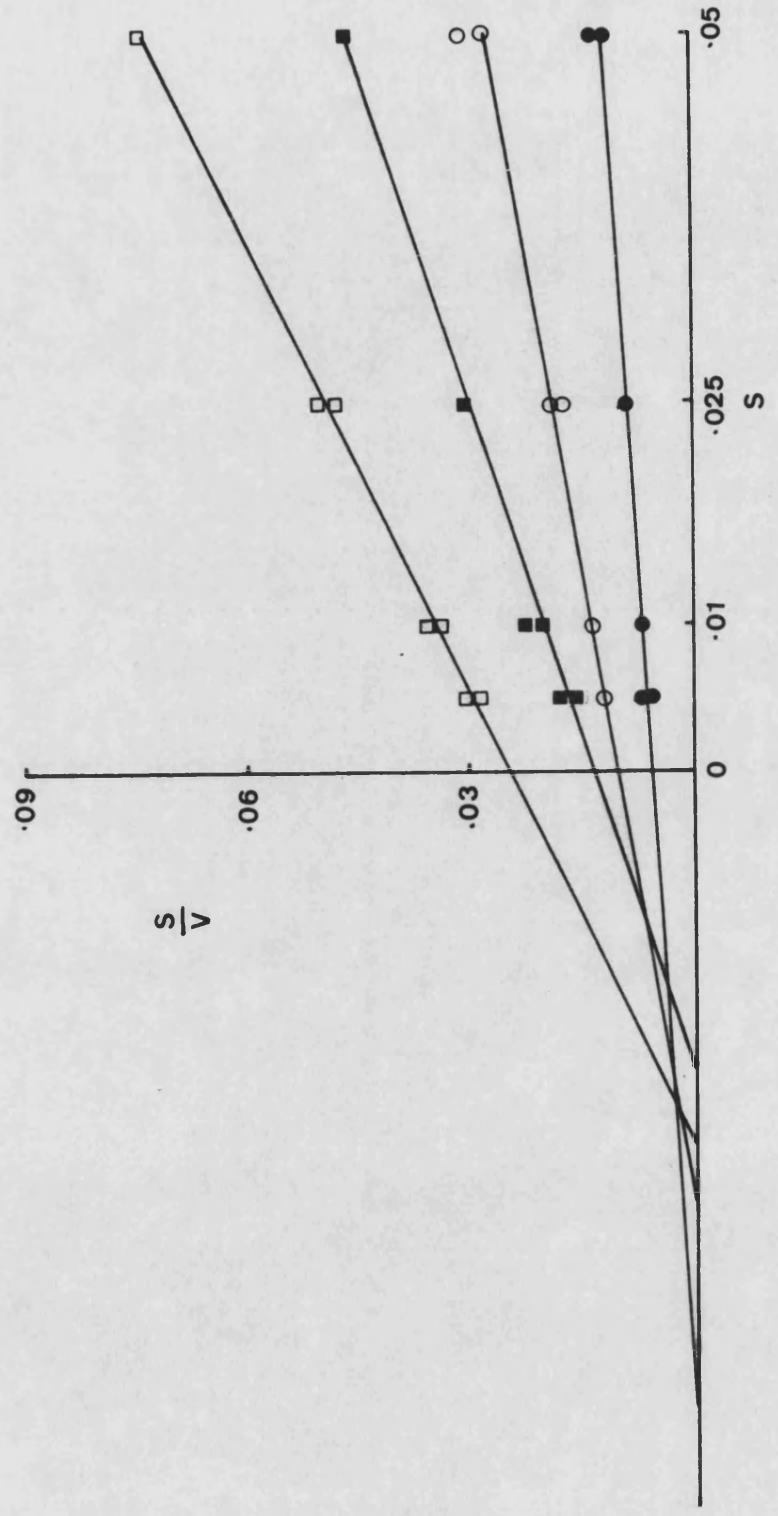

.09
.06
.03
0
.01
.025
.05
S
<V|S

Figure 41: Parameters obtained from the graphical representation of kinetic data obtained

Inhibitor = NAD^+

Fixed substrate = DHAP

Variable substrate = 0.005 - 0.05mM NADH

NAD^+ (mM)	DHAP (mM)	$V_{max(app)}$	$K_{m(app)}$	$\frac{K_{m(app)}}{V_{max(app)}}$
0.0	0.2	0.38	0.0062	0.0163
0.5	0.2	0.41	0.0091	0.0222
5.0	0.2	0.40	0.0200	0.0500
0.0	0.75	1.50	0.0152	0.0101
0.5	0.75	1.57	0.0203	0.0129
5.0	0.75	1.37	0.0367	0.0268
0.0	1.5	2.78	0.0198	0.0071
0.5	1.5	2.75	0.0227	0.0082
5.0	1.5	2.73	0.0526	0.0193
0.0	5.0	8.02	0.0468	0.0058
0.5	5.0	8.54	0.0555	0.0065
5.0	5.0	7.76	0.1027	0.0132

Figure 42: Parameters obtained from the graphical representation of kinetic data obtained

Inhibitor = NAD^+

Fixed substrate = NADH

Variable substrate = 0.2 - 5.0mM DHAP

NAD^+ (mM)	NADH (mM)	$V_{max(app)}$	$K_{m(app)}$	$\frac{K_{m(app)}}{V_{max(app)}}$
0.0	0.005	0.838	0.767	0.9153
0.5	0.005	0.826	1.040	1.2591
5.0	0.005	0.370	0.787	2.1270
0.0	0.01	2.010	1.670	0.8308
0.5	0.01	1.672	1.360	0.8134
5.0	0.01	0.865	1.250	1.4451
0.0	0.025	4.566	2.830	0.6198
0.5	0.025	4.003	2.730	0.6820
5.0	0.025	2.010	1.640	0.8159
0.0	0.05	7.219	3.870	0.5361
0.5	0.05	6.624	3.680	0.5556
5.0	0.05	3.666	2.500	0.6819

Figure 43: Parameters obtained from the graphical representation of kinetic data obtained

Inhibitor = α-GP

Fixed substrate = DHAP

Variable substrate = 0.005 - 0.05mM NADH

α-GP (M)	DHAP (mM)	$V_{max(app)}$	$K_{m(app)}$	$\frac{K_{m(app)}}{V_{max(app)}}$
0.00	0.2	0.410	0.0084	0.0205
0.03	0.2	0.114	0.0076	0.0667
0.06	0.2	0.071	0.0130	0.1831
0.10	0.2	0.047	0.0130	0.2766
0.00	0.75	1.672	0.0210	0.0126
0.03	0.75	0.415	0.0120	0.0289
0.06	0.75	0.219	0.0093	0.0425
0.10	0.75	0.158	0.0130	0.0823
0.00	1.5	3.022	0.0230	0.0076
0.03	1.5	0.836	0.0140	0.0167
0.06	1.5	0.465	0.0140	0.0301
0.10	1.5	0.325	0.0180	0.0554
0.00	5.0	7.331	0.0380	0.0052
0.03	5.0	2.781	0.0280	0.0101
0.06	5.0	1.482	0.0200	0.0135
0.10	5.0	1.021	0.0250	0.0245

Figure 44: Parameters obtained from the graphical representation of kinetic data obtained

Inhibitor = α-GP

Fixed substrate = NADH

Variable substrate = 0.2 - 5.0mM DHAP

α-GP (M)	NADH (mM)	$V_{max(app)}$	$K_{m(app)}$	$\frac{K_{m(app)}}{V_{max(app)}}$
0.00	0.005	0.910	1.10	1.2088
0.03	0.005	0.637	2.63	4.1287
0.06	0.005	0.701	6.08	8.6733
0.10	0.005	0.326	4.87	14.9386
0.00	0.01	2.042	1.61	0.7844
0.03	0.01	1.432	4.40	3.0726
0.06	0.01	1.053	6.69	6.3533
0.10	0.01	0.651	6.57	10.0922
0.00	0.025	4.228	2.46	0.5818
0.03	0.025	3.907	9.35	2.3931
0.06	0.025	2.524	10.96	4.3423
0.10	0.025	1.961	14.19	7.2361
0.00	0.05	7.219	3.95	0.5472
0.03	0.05	4.614	9.21	1.9961

Figure 45: Secondary plot of $K_{m(app)}/V_{max(app)}$ vs (I) from which the K_i value shown in Table 22 was obtained

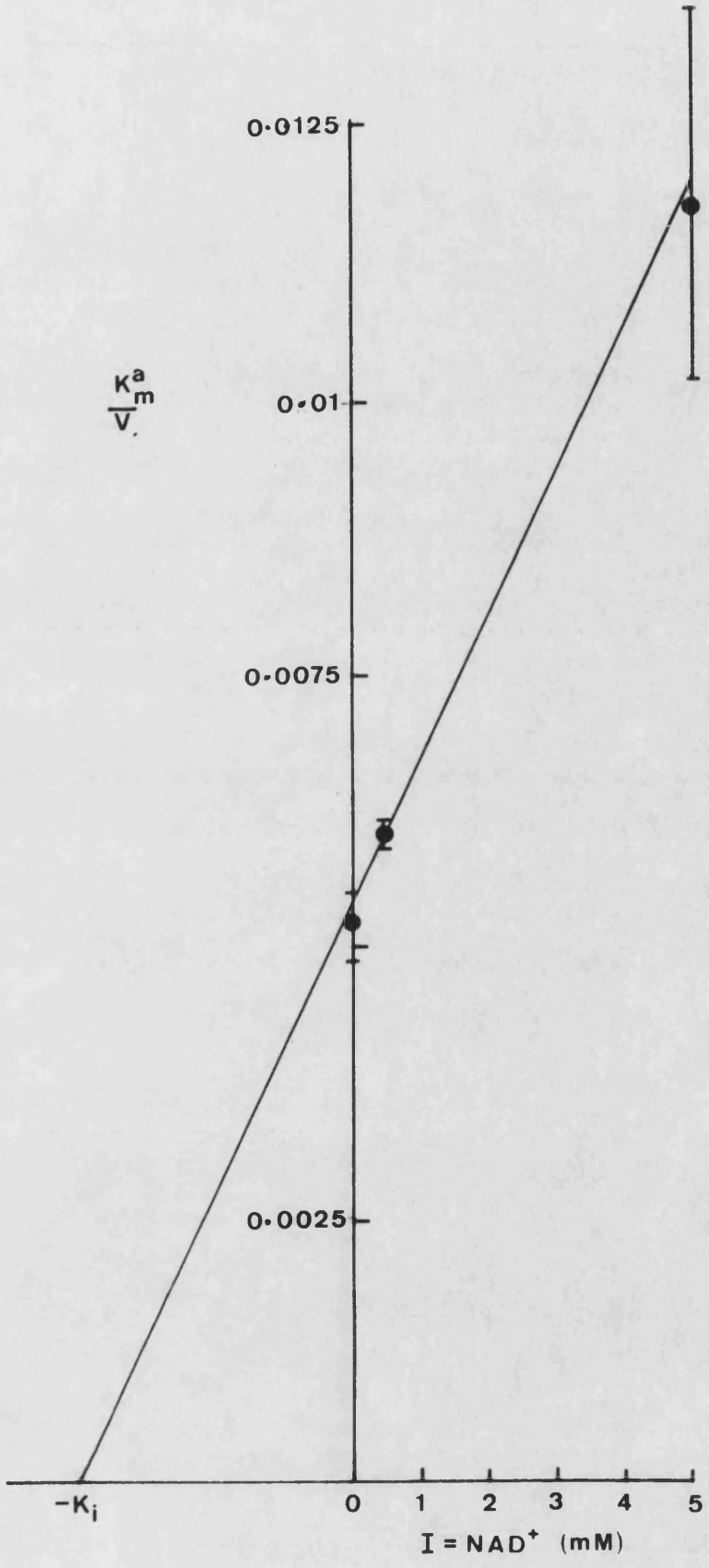

0·0125
0·01
0·0075
0·0025
$\frac{K^a_m}{V}$
$-K_i$
0
1
2
3
4
5
I = NAD+ (mM)

Figure 46: Secondary plot of $K_{m(app)}/V_{max(app)}$ vs (I) from which the K_i value shown in Table 22 was obtained

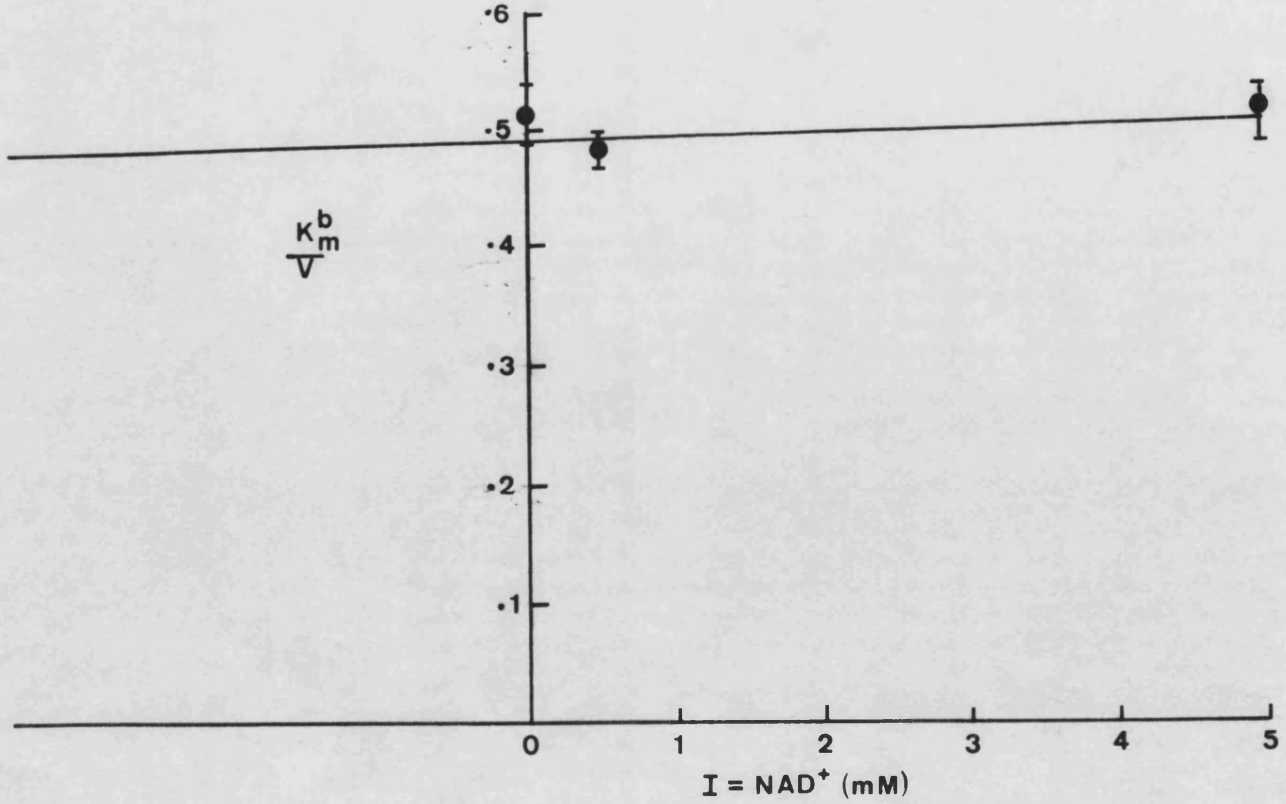

·6
·5
·4
·3
·2
·1
$\frac{K_m^b}{V}$
0
1
2
3
4
5
I = NAD⁺ (mM)

Figure 47: Secondary plot of $K_{m(app)}/V_{max(app)}$ vs (I) from which the K_i value shown in Table 22 was obtained

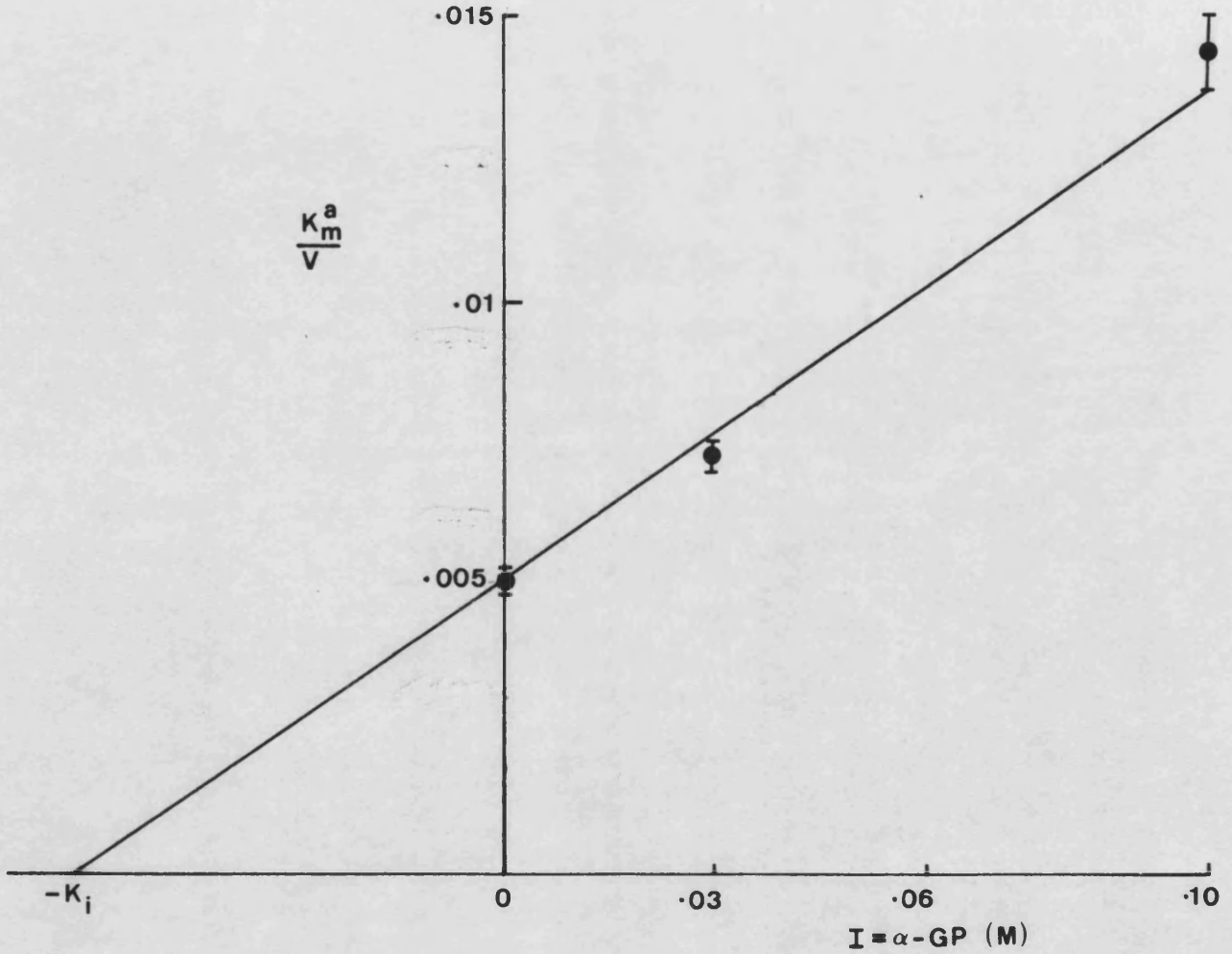
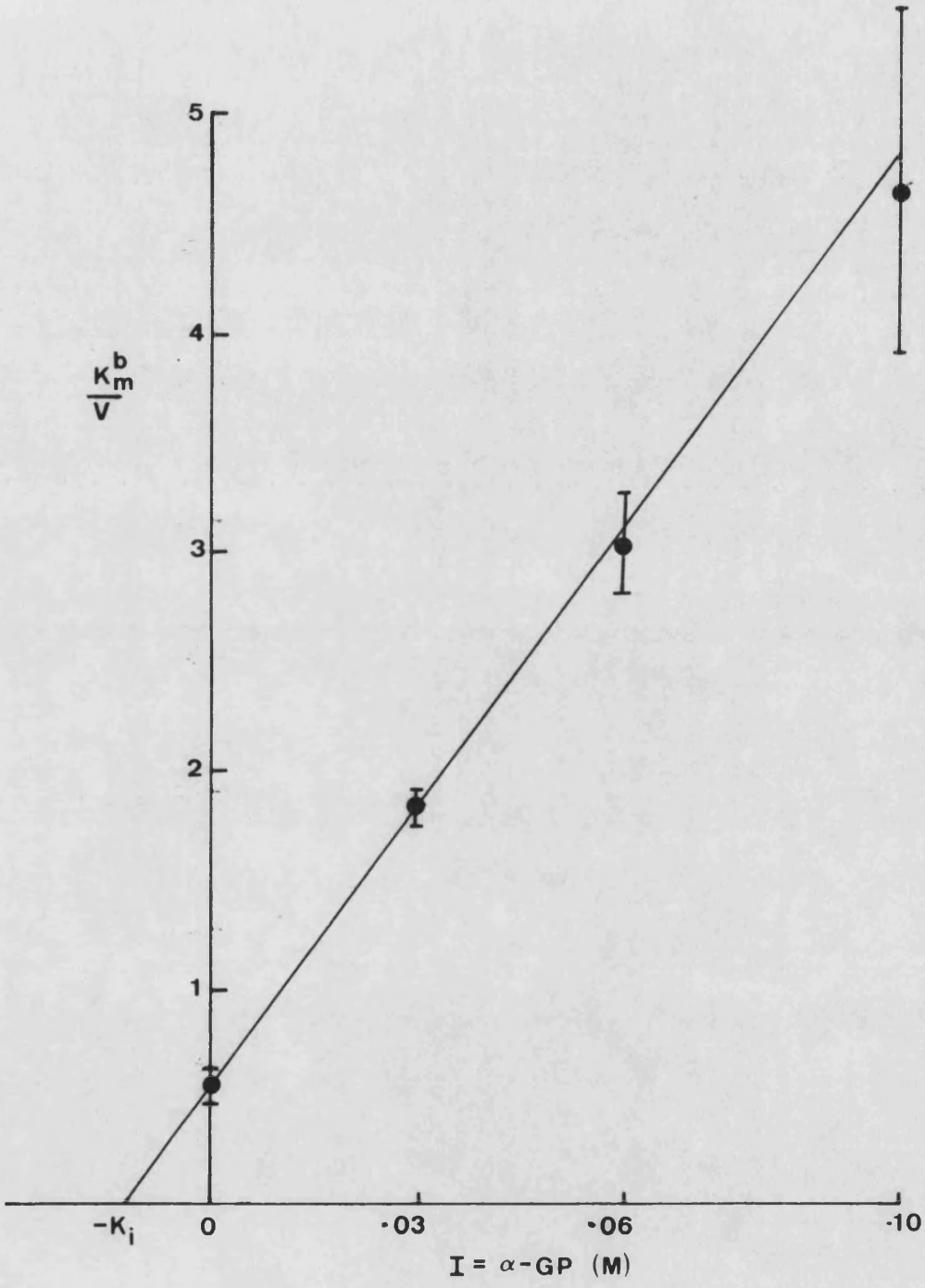

·015
·01
·005
$\frac{K_m^a}{V}$
$-K_i$
0
·03
·06
·10
I = α-GP (M)

Figure 48: Secondary plot of $K_{m(app)}/V_{max(app)}$ vs (I) from which the K_i value shown in Table 22 was obtained


5
4
3
2
1
$\frac{K_m^b}{V}$
$-K_i$
0
·03
·06
·10
I = α-GP (M)

CHAPTER 6: DISCUSSION

6.1: The aerobic/anaerobic transition of glucose metabolism in T. brucei

During the investigation of the aerobic/anaerobic transition of glucose metabolism in Trypanosoma brucei, the data generated experimentally was expressed throughout as the ratio of glycerol produced to pyruvate produced. This means of representation had two advantages over other methods: firstly that the number of trypanosomes present was irrelevant (the counting of the highly-motile cells being subject to large errors) and secondly that, if the widely-held theories of anaerobic metabolism in T. brucei hold true, the glycerol:pyruvate ratio should always have been unity or less. Such values were anticipated as simple to represent graphically and also sufficiently 'managable' for comparisons easily to be made.

During the transition studies a glycerol:pyruvate ratio of at least 0.07 was consistently found in the incubation mixture, even under aerobic conditions. Investigation of the available literature revealed that this was also the case for the majority of other workers, among them Ryley (1956), Flynn and Bowman (1973) and MacKenzie et al. (1982). The presence of glycerol was attributed to 'transient anaerobic conditions' arising during the handling of the trypanosomes by Fairlamb and Bowman (1980a) and was dismissed as insignificant by the same authors in another piece of work published in the same year (1980b). Other workers merely ignore the production of glycerol during the incubation of trypanosomes under aerobic

conditions, assuming equimolar pyruvate and glycerol to be produced within experimental error. It has been shown in this work that the production of a level of glycerol slightly higher than that predicted was not only seen under anaerobic conditions but also aerobic conditions and at every measured oxygen tension between these two extremes. That this glycerol production was not due to transient anaerobic conditions being experienced by the trypanosomes during handling can be proven by the following calculation:

Glycerol is theoretically produced anaerobically at a rate of 5μmol/hour/1 x 10^8 cells (Brohn and Clarkson, 1980). The time-lapse between removal of a sample of incubation medium and rendering it cell-free prior to freezing was a maximum of two minutes. The amount of glycerol produced in two minutes in a 1ml volume at 2 x 10^7 cells/ml would be approximately 0.033μmol. The actual amount of glycerol consistently found over and above that predicted was between 0.1 and 0.2μmol/ml and so, although transient anaerobiosis may have occurred during handling, it was certainly not responsible for the entire amount of glycerol produced.

A second reason for the apparent excess of glycerol produced could have been that the glycerol used to make up standard solutions was less than 100% pure, as glycerol takes up water with time. In order to determine its purity, the refractive index of the stock glycerol was measured using an Abbé refractometer (Hilger and Watts, London). Reference to a table of refractive indices of glycerol/water solutions (Newman, 1968) showed that the stock solution was in fact

98% glycerol by weight, leading to an excess of only 0.02 in any glycerol:pyruvate value. The excess actually observed was approximately 0.1 and the slight impurity of the stock glycerol could not account for this.

It appears then that there may exist some alternative pathway, as yet unknown, which is responsible for the production of a small but significant quantity of glycerol and which operates at all oxygen tensions.

As stated in Section 3.2.2, the value of 'K' obtained from the model used was 3.6 $\pm$ 0.8mmHg, corresponding to an oxygen concentration of 5.0μM. The K_m for oxygen of trypanosomal α-glycerophosphate oxidase in intact cells has been estimated at between 2 and 8μM (Hill, 1976a, 1976b). Although there are difficulties in the interpretation of K_m values of intact cells (Fisher, 1964), the two values are in sufficiently good agreement for the proposal of α-glycerophosphate oxidase as the key enzyme in the aerobic/anaerobic transition of glucose metabolism in T. brucei.

The second important point arising from the investigation of the aerobic/anaerobic transition of glucose metabolism in T. brucei is apparent from Figure 15. The oxygen tension of human blood is widely-quoted as 40mmHg for venous blood and 100mmHg for arterial blood (Cantarow and Trumper, 1962; Green, 1978) and it can be seen that between these two values the anaerobic pathway is inoperative, the glycerol:pyruvate ratio remaining constant at approximately 0.1. While the implication of this is that the anaerobic pathway has little or no physiological significance in the bloodstream of the mammalian host, it is possible that oxygen concentrations lower than those

quoted above do in fact exist. For example, von Brand (1966) states that 'the mere presence of parasites may materially influence the local oxygen tension since it has been established that inflammatory processes lower the oxygen tension'. Also of course, in the later stages of infection, when there is CNS involvement, trypanosomes invade the cerebrospinal fluid where oxygen concentrations may be considerably lower than those of the bloodstream of a normal, healthy person.

6.2: The purification of trypanosomal α-GPDH

It could reasonably be argued that a study of the kinetic properties of an enzyme would best be carried out on a crude cell preparation, this being a more realistic representation of the *in vivo* behaviour of an enzyme than is a purified preparation. That the results obtained may differ has been demonstrated by Cronin and Tipton (1985) when comparing their work on purified phosphofructokinase from *T. brucei* with that of Nwagwu and Opperdoes (1982) who used a crude cell extract. Similarly the kinetic values reported here and obtained from experiments on a purified extract of α-GPDH from *T. brucei* are significantly different from those obtained by Reynolds (1975) who used a crude cell-lysate. As to the relative merits of the two sources of the enzyme, Misset and Opperdoes (1984) stated that 'detailed differences in the physical, chemical and structural properties of the parasite and host glycolytic enzymes are an essential prerequisite for the successful development of anti-glycolytic compounds. Such differences can only be identified by

using purified enzymes'. As the ultimate aim of most work carried out in this field is the identification and development of some trypanocidal drug, it was decided that the experiments described here should be carried out on purified α-GPDH. The enzyme from T. brucei had not previously been purified and it was therefore necessary to devise a method for doing so.

While Opperdoes (1985) may consider that T. brucei has almost become the Escherichia coli of biochemical parasitology in being used as a 'model organism', it in no way represents E. coli in the ease of obtaining cellular material quickly and in large quantities. Attempts have been made to discover a system whereby T. brucei may be grown in tissue culture (Hirumi et al., 1980; Brun et al., 1984). Efforts on this theme continue but until a satisfactory method is perfected, yielding high cell numbers and densities, the use of laboratory animals for the growth of trypanosomes must continue in the face of increasing public opposition and with the associated inadequate cell yield.

The attempt to purify α-GPDH from T. brucei began with the isolation of a glycosomal fraction, it being widely-accepted as primarily a glycosomal enzyme (for example, Opperdoes et al. (1977) and Misset et al. (1986)), although found mainly in the cytoplasm of mammalian cells (Baranowski, 1963). Following the development of an optimal method for the preparation of a glycosomal fraction, various techniques routinely used in the purification of enzymes were attempted, but most ended in failure.

Gel filtration, a quick and simple method commonly used in the purification of protein mixtures, was anticipated to be a useful step

early in the procedure. The molecular weight of each subunit of trypanosomal α-GPDH has been determined as lying between 37,000 (Hart et al, 1984) and 38,900 (Aman et al, 1985), all workers agreeing that the enzyme is dimeric, yielding molecular weight values of between 74,000 and 77,800. Armed with this knowledge, gels with a suitable pore-size were selected and several different columns run under various conditions (see Section 4.7). Very little α-GPDH activity was recovered from the columns and it was calculated that approximately 8% of the applied protein was not eluted from the column. Since these attempts, Misset et al. (1986) have reported similar findings, concluding that α-GPDH (and also 6-phosphofructokinase) was retained on the column because of electrostatic interaction with the matrix. Furthermore, they have discovered that the trypanosomal glycolytic enzymes are all basic proteins, α-GPDH having a pI value of approximately 10.0. Approximate values for pI of α-GPDH from other sources include 6.4 for the rabbit-muscle enzyme (Misset et al, 1986) and 5.3 for α-GPDH obtained from Drosophila melanogaster (Bewley et al, 1984). The above values indicate that at neutral pH α-GPDH from T. brucei (and similarly the other glycosomal enzymes) carries a net positive charge while that from other species has a net negative charge. Misset et al. (1986) suggest that the positive charges of the trypanosomal proteins may play an important role in the entry of the enzymes into the glycosome. Such a marked difference in a physical property of the enzymes from the trypanosome and from potential hosts may be a starting-point for the development of new, specific anti-trypanosomal drugs.

Misset and Opperdoes (1984) reported difficulties with 'salting-out' of glycosomal enzymes from T. brucei using ammonium sulphate nor had they any success with affinity chromatography using immobilised NAD^+, being completely unable to elute any of the enzymes by any method they attempted. Affinity chromatography methods and ammonium sulphate precipitation were attempted during this work (Sections 4.4 and 4.9 respectively) and were similarly unsuccessful. As detailed in Section 4.8, efforts to purify α-GPDH by ion-exchange chromatography also met with little success, incomplete adsorption occurring with both anion- and cation-exchangers. These observations could be an effect of the unusually high pI of trypanosomal α-GPDH combined with the conditions used, although there is a possibility that more than one form of the enzyme may exist, as reported by McLaughlin and MacQuarrie (1978) for the enzyme from various species including chickens, humans and rats.

The failure of non-denaturing PAGE electrophoresis as a separation method (Section 4.11.6) has also been experienced by Cronin and Tipton (1985) with respect to trypanosomal phosphofrucokinase. Staining methods devised for use during this technique (Section 4.11.5) tentatively identified the presence of a multi-enzyme complex too large to penetrate the gel. Although the existence of such a complex within the glycosome of T. brucei has been investigated (Oduro et al., 1980a,b; Aman et al., 1985) its presence in vivo remains in question (Misset et al., 1986).

The purification scheme eventually used gave a low yield although the quantity, activity and stability of the pure enzyme was more than adequate for kinetic studies to be carried out. The difficulties

encountered during the development of a purification scheme for trypanosomal α-GPDH were unexpected and seem to bear out the opinion of Misset and Opperdoes (1984) who, faced with similarly frustrating results, concluded that they typified 'the bizarre properties of the trypanosomal enzymes, for which as yet no explanation can be given'. With hindsight, a more thorough investigation of the physical, chemical and structural properties of trypanosomal α-GPDH prior to the commencement of purification would have been of considerable assistance in the choice of separation techniques.

6.3: The kinetic behaviour of trypanosomal α-GPDH

The kinetic studies presented here were restricted throughout by the practical impossibility of assaying α-GPDH in the presence of saturating concentrations of NADH, the spectrophotometric system used being unable to record such potentially high absorbance values. The widely-available tables for the deduction of enzyme mechanism generally require the type of inhibition seen in the presence of saturating substrate concentrations to be known (Wharton and Eisenthal, 1981 for example), and consequently such tables could not be used to their fullest extent. However, as described in Section 5.2.2, the action of trypanosomal α-GPDH seems to be by means of a compulsory-order mechanism with NADH binding to the enzyme first and NAD^+ being released from the enzyme last. Although the published diagnostic tables could not be used to their full advantage, it seems that the compulsory-order mechanism is the most likely, since 'it appears that the compulsory binding order mechanism may be a rather

general one for enzymes reacting with diphosphopyridine nucleotides' (Raval and Wolfe, 1962).

In order to determine whether α-GPDH would be operative under conditions of anaerobic respiration, it was first necessary to ascertain intracellular levels of the relevant metabolites under both aerobic and anaerobic conditions. The calculation of these concentrations was complicated by differing opinions as to the precise location of the metabolites. Opperdoes and Borst (1977) proposed that the glycosomal membrane is impermeable to glycolytic intermediates while Visser et al. (1981) stated that between 20 and 30% of total cellular metabolites are contained within the glycosome and this value has been widely-accepted (Misset and Opperdoes, 1984; Aman et al., 1985). Recently however, Patthey and Deshusses (1987) have suggested that the glycosomal membrane is freely permeable to small molecules, the size of metabolites, but impermeable to larger molecules. Consequently the concentrations of NAD^+, NADH, α-GP and DHAP under aerobic and anaerobic conditions were calculated as shown in Figures 49, 50 and 51 and the results shown in Table 24 below.

As stated in Section 5.2.2, the rate equation for α-GPDH in the absence of inhibitors is:

$$v = \frac{V_{max}}{1 + \frac{K_{ma}}{(A)} + \frac{K_{mb}}{(B)} + \frac{K_{ab}}{(A)(B)}}$$

Substituting with values previously obtained (Figure 30 and Table 24) the theoretical maximum velocity of the enzyme aerobically and in the absence of inhibitors is calculated as 0.0106mmol/minute/1 x 10^8 cells in the situation where 25% of glycolytic intermediates are contained in the glycosome. The same calculation, with values for the concentrations of metabolites if confined solely to the glycosome and freely distributed throughout the cell yield theoretical maximum velocities of 0.0232 and 0.0022mmol/minute/1 x 10^8 cells respectively.

In the presence of inhibitors the rate equation can be expanded to:

$$v = \frac{V_{max}}{1 + \frac{K_{ma}}{(NADH)}\left[1 + \frac{(NAD^+)}{K_{i2}}\right]\left[1 + \frac{(\alpha\text{-GP})}{K_{i4}}\right] + \frac{K_{mb}}{(DHAP)}\left[1 + \frac{(\alpha\text{-GP})}{K_{i3}}\right] + \frac{K_{ab}}{(NADH)(DHAP)}}$$

As a value for V_{max} could not accurately be estimated in the presence of inhibitors, these results are best represented thus:

$$\frac{v}{V_{max}} = \frac{1}{1 + \frac{K_{ma}}{(NADH)}\left[1 + \frac{(NAD^+)}{K_{i2}}\right]\left[1 + \frac{(\alpha\text{-GP})}{K_{i4}}\right] + \frac{K_{mb}}{(DHAP)}\left[1 + \frac{(\alpha\text{-GP})}{K_{i3}}\right] + \frac{K_{ab}}{(NADH)(DHAP)}}$$

Then, substituting the values above with those obtained for the aerobic condition when 25% of metabolites are contained within the glycosome (Table 24), the calculation yields a v/V_{max} value of 0.233. Substituting similarly for the anaerobic condition and also for the situations when the glycolytic intermediates are confined to the glycosome and when the membrane is freely permeable, the values shown in Figure 25 are obtained.

These values are in fact likely to be over-estimated as no account has been taken of the reverse reaction which must occur to some extent in the presence of α-GP and NAD^+. The estimated velocities at which trypanosomal α-GPDH can operate in vivo may be calculated, since the theoretical maximum velocity at which the enzyme can operate under aerobic and anaerobic conditions is known (see above). The values obtained are as shown in Table 26.

Table 24: The concentrations of some trypanosomal glycolytic intermediates under aerobic and anaerobic conditions

Metabolite	Aerobic (mM)	Anaerobic (mM)
NAD^+: A	0.158	0.044
B	0.920	0.255
C	3.690	1.020
NADH: A	0.070	0.184
B	0.405	1.070
C	1.620	4.290
DHAP: A	1.043	0.448
B	6.089	2.617
C	24.400	10.480
α-GP: A	0.767	3.102
B	4.480	18.120
C	17.900	72.600

where A represents the situation where the glycosomal membrane is

freely permeable to metabolites, B the situation where 25% of the total cellular metabolites are located within the glycosome and C where metabolites are completely confined within the glycosome.

Table 25: The proportion of the maximum uninhibited velocity of trypanosomal α-GPDH at which the enzyme may operate in the presence of products as inhibitors

Glycolytic intermediates	Aerobically	Anaerobically
	v/V_{max}	
confined to glycosome	0.390	0.099
25% in glycosome	0.223	0.071
freely distributed	0.056	0.024

Table 26: The estimated velocities at which trypanosomal α-GPDH may operate in vivo

Glycolytic intermediates	Aerobically	Anaerobically
	μmol/minute/1x10^8 cells	
confined to glycosome	9.048	2.297
25% in glycosome	2.364	0.753
freely permeable	0.123	0.053

As previously stated (Section 1.8), the rate of glycolysis in Trypanosoma brucei lies between 0.041 and 0.082μmol glucose consumed/minute/1 x 10^8 cells with a resultant NADH flux of 0.082-0.164μmol/minute/1 x 10^8 cells under aerobic conditions and 0.041-0.082μmol/minute/1 x 10^8 cells anaerobically. From the values shown in Table 26, it is apparent that the enzyme is well able to operate under these conditions.

The values obtained for the kinetic constants of trypanosomal α-GPDH in the absence of inhibitors (Section 5.2.1) are compared with

those of the enzyme from other species in Table 27 below. The values for chicken liver α-GPDH were obtained from Walsh and Sallach (1965) and for rabbit muscle from Barman (1969).

Table 27: Comparison of the kinetic parameters for α-GPDH from T. brucei with those from other sources

Source	Substrate	K_m (M)	Conditions
Trypanosoma brucei	NADH	1.25×10^{-4}	pH 7.4, 37.0°C, phosphate buffer
	DHAP	1.36×10^{-2}	
Rabbit muscle	NADH	-	pH 7.0, 23.3°C, phosphate buffer
	DHAP	4.60×10^{-4}	
Chicken liver	NADH	5.00×10^{-6}	pH 8.0, 25°C, tris buffer
	DHAP	1.00×10^{-5}	

It is apparent that the K_m values for T. brucei are considerably larger than those for mammalian cells. The cytosolic concentration of DHAP in rat heart muscle is 27.5μM (Reynolds et al., 1971) and the ratio of α-GP:DHAP in the same tissue is approximately 2.4 (Garland et al., 1964), thus the concentration of α-GP may be calculated as 66μM. When these values are compared with those calculated for T. brucei in Table 24, it can be seen that the intracellular concentrations of α-GP and DHAP are many times greater in T. brucei than in the mammalian tissue and the values for K_m may reflect this.

6.4: Future work and general conclusions

As yet no method exists for the isolation of functional glycosomes. The development of such a technique and conditions for their incubation would be beneficial in the elucidation of the glycolytic pathway of T. brucei. Similarly useful and interesting would be an investigation into possible alternative pathways for the production of the small but significant amount of glycerol consistently seen during experimentation on metabolising cells.

The implication that the anaerobic pathway of glucose metabolism may not be operative in vivo (Section 6.1) is both interesting and unexpected while the work discussed in Section 6.3 seems to disprove the hypothesis that the high level of α-GP found in T. brucei under anaerobic conditions inhibits the action of α-GPDH while enhancing that of glycerol kinase as it has been shown here that trypanosomal α-GPDH is capable of operating at the required rate anaerobically.

Fairlamb (1982) stated that 'rational drug design is still in its infancy, partly because fundamental knowledge of many areas of the parasites' metabolism is still lacking'. Although the work described here seems to have no immediate benefit, the conclusions drawn from it could prove useful to future workers in the search for safe and effective trypanocidal drugs.

Figure 49: The calculation of intraglycosomal concentrations of NAD^+ and NADH

1) Alsharif *et al.* (1986) have estimated that the total cellular concentration of NAD/NADH is 13.2 ± 2.5pmol/1 x 10^6 cells.

Visser and Opperdoes (1980) have determined that the ratio NADH:NAD+ is 0.44 aerobically and 4.2 anaerobically.

The total cellular concentration is 1.32mmol/1 x 10^8 cells and so, assuming that the metabolite is freely distributed throughout the trypanosome (Patthey and Deshusses, 1987), the required concentrations would be:

Aerobically NAD^+ = 0.917mmol/1 x 10^8 cells

NADH = 0.403mmol/1 x 10^8 cells

Anaerobically NAD^+ = 0.254mmol/1 x 10^8 cells

NADH = 1.066mmol/1 x 10^8 cells

Opperdoes *et al.* (1984) have determined the volume of a single trypanosome to be 58μm^3. Incorporation of this value into the above calculations yields values as follows:

Aerobically NAD^+ = 0.158mM

NADH = 0.070mM

and anaerobically NAD^+ = 0.044mM

NADH = 0.184mM.

2) If NADH and NAD^+ were confined to the glycosome as suggested by Opperdoes and Borst (1977) then the calculation would be as described below:

The volume of one glycosome is $0.0108\mu m^3$ and the average trypanosome contains approximately 230 glycosomes (Opperdoes et al., 1984). The total glycosomal volume is therefore $2.484 \times 108\mu m^3/1 \times 10^8$ cells.

The concentration of NAD^+ within the glycosome aerobically would thus be:

$$0.917\text{nmol}/2.474 \times 10^8 \mu m^3 = 3.69\text{mM}$$

and of NADH would be:

$$0.403\text{nmol}/2.484 \times 10^8 \mu m^3 = 1.62\text{mM}.$$

Anaerobically the values would become 1.02mM for NAD^+ and 4.29mM for NADH.

3) The third possibility, proposed by Visser et al. (1981) is that approximately 25% of the total cellular metabolites are situated within the glycosome. The intraglycosomal concentrations would then become:

Aerobically NAD^+ = 0.92mM

NADH = 0.405mM

and anaerobically NAD^+ = 0.255mM

NADH = 1.070mM.

Figure 50: The calculation of intraglycosomal concentrations of α-GP

1) Visser and Opperdoes (1980) state that the intracellular concentrations of α-GP are 0.89μmol/g wet weight aerobically and 3.60μmol/g wet weight anaerobically. It has been calculated during this work that 1g wet weight represents approximately 2×10^{10} cells. For the case where α-GP is freely distributed throughout the cell (Patthey and Deshusses, 1987), the volume of 1 trypanosome is taken as $58\mu m^3$ (Opperdoes et al., 1984). Thus the concentration of α-GP aerobically is,

$0.89\mu mol/2 \times 10^{10}$ cells

$= 0.89\mu mol/2 \times 10^{10} \times 58\mu\ m^3$

$= 0.89\mu mol/1.16 \times 10^{-3}$ litre

$= 767\mu mol/litre$

$= 0.767mM.$

and similarly, anaerobically;

$3.60\mu\ mol/2 \times 10^{10}$ cells

$= 3.102mM.$

2) If α-GP was confined to the glycosome, as proposed by Opperdoes and Borst (1977) the calculation would become, aerobically;

$0.89\mu mol/2 \times 10^{10}$ cells

$= 0.89\mu mol/2 \times 10^{10} \times 0.0108 \times 230\mu m^3$

$= 17.900mM$

and anaerobically

$3.60\mu mol/2 \times 10^{10}$ cells

$= 72.600mM.$

3) In the situation where only 25% of total α-GP is confined within the glycosome, the intraglycosomal concentration becomes approximately 4.480mM aerobically and 18.120mM anaerobically.

Figure 51: The calculation of intraglycosomal concentrations of DHAP

1) Visser and Opperdoes (1980) determined that the intracellular concentration of DHAP is 1.21μmol/g wet weight aerobically and 0.52μmol/g wet weight anaerobically. As 1g wet wieght has been estimated as 2×10^{10} cells and the volume of one trypanosome as $58\mu m^3$, the concentration of DHAP if freely distributed throughout the cell (Patthey and Deshusses, 1987) would be:

$$\text{Aerobically DHAP} = 1.21\mu mol/2 \times 10^{10} \times 58\mu m^3$$
$$= 1.043mM$$

$$\text{and anaerobically DHAP} = 0.52\mu mol/2 \times 10^{10} \times 58\mu m^3$$
$$= 0.448mM.$$

2) If DHAP were confined to the glycosome as suggested by Opperdoes and Borst (1977), the calculation would become:

$$\text{Aerobically DHAP} = 1.21\mu mol/2 \times 10^{10} \times 230 \times 0.0108\mu m^3$$
$$= 24.400mM$$
$$\text{and anaerobically DHAP} = 0.52\mu mol/2 \times 10^{10} \times 230 \times 0.0108\mu m^3$$
$$= 10.48mM$$

if the volume of one glycosome is $0.0108\mu m^3$ and there are an average of 230 per cell (Opperdoes et al., 1984).

3) If, as suggested by Visser et al. (1981), 25% of the total cellular DHAP is confined to the glycosome, the intraglycosomal concentrations become 6.089mM and 2.617mM aerobically and anaerobically respectively.

References

Abolarin, M.O., Evans, D.A., Tovey D.G. and Ormerod, W.E., (1982), British Medical Journal, 285, 1380 - 1382.

Alsharif, M., Whish, W.D.J., Farzaneh, F. and Eisenthal, R., (1986), Biochemical Society Transactions, 14, 105 - 106.

Amole, B.O. and Clarkson, A.B.,Jr., (1981), Experimental Parasitology, 51, 133 - 140.

Aman, R.A., Kenyon, G.L. and Wang, C.C., (1985), J. Biol. Chem., 260(II), 6966 - 6973.

Bacchi, C.J., Marcus, S.L., Lambros, C., Goldberg, B., Messina, L. and Hutner, S.H., (1974), Biochem. Biophys. Res. Comm., 58(3), 778 - 786.

Baranowski, T., (1963), in "The Enzymes", Boyer, P.D., Lardy, H. and Myrback, K. (eds), 7, 2nd edn., Academic Press, London, 85 - 96.

Barman, T.E., (1969), "The Enzyme Handbook", I, Springer-Verlag, Berlin, 32.

Bensadoun, A. and Weinstein, D., (1976), Analyt. Biochem., 70, 241 - 250.

Bergmeyer, H.U. (ed), (1974), "Methods in Enzymatic Analysis", 2nd edn., Academic Press, London.

Bird, R.G., Ellis, D.S., Ormerod, W.E., Parr, C.W., Venkatesan, S. and Welch, S.G., (1971), Comp. Biochem. Physiol, 40(B), 481 - 487.

Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S., (1973), Archs Biochem. Biophys., 155, 213 - 220.

Boreham, P.F.L., (1979), in "Biochemistry and Physiology of Protozoa", Levandowsky, M. and Hutner, S.H. (eds), 2, Academic Press, London, 429 - 457.

Bowman, I.B.R. and Flynn, I.W., (1976), in "Biology of the Kinetoplastida", Lumsden, W.K.R.(ed), I, Academic Press, London.

Bradford, M.M., (1976), Analyt. Biochem., 72, 248 - 254.

Brohn, F.H. and Clarkson, A.B.Jr., (1978), Acta Tropica, 35, 23 - 33.

Brohn, F.H. and Clarkson, A.B.Jr., (1980)I, Molec.Biochem. Parasitol., 291 - 305.

Brown, R.C., Evans, D.A. and Vickerman, K., (1973), Int. J. Parasitol., 3(5), 691 - 704.

Brun, R., Grootenhuis, J.C., Kunz, C. and Schonenberger, M., (1984),

J. Parasitol., 70(5), 836 - 837.

Bursell, E., (1966), Comp. Biochem. Physiol., 19, 809 - 812.

Cantarow, A. and Trumper, M., (1962), in "Clinical Biochemistry", 6th edn., W.B. Saunders, Philadelphia.

Chappell, L.H., (1980), "Physiology of Parasites, Blackie, London.

Clarkson, A.B.Jr. and Brohn, F.H., (1976), Science N.Y., 194, 204 - 206.

Colowick, S.P., (1955), in "Methods in Enzymology", Colowick, S.P. and Kaplan, N.O. (eds), I, Academic Press, London.

Cornish-Bowden, A. and Eisenthal, R., (1978), Biochim. Biophys. Acta, 523, 268 - 272.

Cox, F.E.G. (ed), (1982), "Modern Parasitology", Blackwell Scientific Publications, London.

Cronin, C.N. and Tipton, K.F., (1958), Biochem. J., 227(1), 113 - 124.

Cross, G.A.M. and Johnson, J.G., (1976), in "Biochemistry of Parasites and Host-Parasite Relationships", van den Bossche (ed), Elsevier/North-Holland Biomedical Press, Amsterdam.

Damper, D. and Patton, C.L., (1976), Biochem. Pharmacol., 25, 271 - 276.

Dewey, M.M. and Conklin, J.L., (1960), Proc. Soc. exp. Biol. Med., 105, 492 - 494.

Davis, B.J., (1964), Ann. N.Y. Acad. Sci., 121, 404 - 427.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M., (1969), "Data for Biochemical Research", 2nd edn., Clarendon Press, Oxford.

Dixon, H., (1966), Nature, 210, 428.

Dixon, M. and Webb, E.C., (1979), "Enzymes", 3rd edn., Longman Group Ltd., London.

Donaldson, R.J. (ed), (1979), "Parasites and Western Man", MTP Press Ltd., Lancaster.

Donelson, J.E. and Turner, M.J., (1985), Scientific American, 252(2), 32 - 39.

Eisenthal, R. and Cornish-Bowden, A., (1974), Biochem. J., 139, 715 - 720.

Evans, D.A., Brightman, C.J. and Holland, M.F., (1977), Lancet, 2, 769.

Evans, D.A. and Brown, R.C., (1973), J. Protozool., 20, 157 - 160.

Faires, R.A. and Parkes, B.H., (1964), in "Radioisotope Laboratory Techniques", George Newnes Ltd., London.

Fairlamb, A., (1982), TIBS, 7, 249 - 252.

Fairlamb, A.H. and Bowman, I.B.R., (1977a), Int. J. Biochem., 8, 659 - 668.

Fairlamb, A.H. and Bowman, I.B.R., (1977b), Int. J. Biochem., 8, 669 - 675.

Fairlamb, A.H. and Bowman, I.B.R., (1980a), Exp. Parasitol., 49, 366 - 380.

Fairlamb, A.H. and Bowman, I.B.R., 1(1980b), Mol. Biochem. Parasitol., 315 - 333.

Fairlamb, A.H., Opperdoes, F.R. and Borst, P., (1977), Nature, 265, 270 - 271.

Ferguson, M.A.J., Low, M.G. and Cross, G.A.M., (1985), J. Biol.Chem., 260(27), 14547 - 14555.

Fisher, R.B., (1964), "Oxygen in the Animal Organism", Pergamon Press, London.

Flynn, I.W. and Bowman, I.B.R., (1973), Comp.Biochem. Physiol., 45B, 25 - 42.

Flynn, I.W. and Bowman, I.B.R., (1974), Comp. Biochem. Physiol., 48B, 261 - 273.

Gabriel, O., (1971), Meth. Enzymol., XXII, 578 - 604.

Garland, P.B., Newsholme, E.A. and Randle, P.J., (1964), Biochem. J., 93, 665 - 678.

Goodwin, L.G., (1985), Br. Med. Bull., 41(2), 103 - 104.

Gordon, A.H., (1975), "Electrophoresis of Proteins in Polyacrylamide and Statch Gels", North-Holland/American Elsevier, 169s - 170s.

Grant, P.T. and Sargent, J.R., (1960), Biochem. J., 76, 229 - 237.

Grant, P.T., Sargent, J.R. and Ryley, J.F., (1961), Biochem. J., 81, 200 - 206.

Green, J.H., (1978), "Basic Clinical Physiology", 3rd edn., Oxford Universtiy Press, Oxford.

Gruenberg, J., Schwendimann, B., Sharma, P.R. and Deshusses, J., (1980), J. Protozool., 27(4), 484 - 491.

Gutteridge, W.E., (1985), Br. Med. Bull., 41(2), 162 - 168.

Hames, B.D. and Rickwood, D. (eds), (1981), "Gel electrophoresis of Proteins: a Practical Approach", IRL Press Ltd., Oxford.

Hammond, D.J., Aman, R.A. and Wang, C.C., (1985), J. biol. Chem., 269(29), 15646 - 15654.

Hammond, D.J. and Bowman, I.B.R., 2(1980a), Mol. Biochem. Parasitol., 63 - 75.

Hammond, D.J. and Bowman, I.B.R., 2(1980b), Mol. Biochem. Parasitol., 77 - 91.

Hart, D.T.. Misset, O., Edwards, S.W. and Opperdoes, F.R., (1984), Mol. Biochem. Parasitol., 12, 25 - 35.

Harvey, M.J., Lowe, C.R. and Dean, P.D.G., (1974), Eur. J. Biochem., 41, 353 - 357.

Hill, G.C., (1976a), Biochim. Biophys. Acta, 456, 149 - 193.

Hill, G.C., (1976b), in "Biochemistry of Parasites and Host-Parasite Relationships', van den Bossche, H. (ed), Elsevier/North-Holland Biomedical Press, Amsterdam, 31 - 50.

Hirumi, H., Hirumi, K., Doyle, J.J. and Cross, G.A.M., (1980),

Parasitol., 80, 371 - 382.

Hoare, C.A., (1964), J. Protozool., 11(2), 200 - 207.

Hoare, C.A. and Wallace, F.G., (1966), Nature, 212, 1385 - 1386.

Howells, R.E., (1985), Parasitol., 90, 687 - 703.

Ingham, K.C., (1984), Meth. Enzymol., 104, Academic Press, London, 351 - 356.

Jenni, L., Marti, S., Schwezer, J., Betschart, B, le Page, R.W.F., Wells, J.M., Tait, A., Paindevoine, P., Pays, E. and Steinert, M., (1986), Nature, 322, 173 - 175.

Jordan, A.M., (1985), Brit. Med. Bull., 41(2), 181 - 186.

Keston, A.S., (1956), Abstract of Papers, 129th Meeting, ACS, Dallas (Tx), 310.

Kilgour, V., (1980), Mol. Biochem. Parasitol., 2, 51 - 62.

Kolata, G., (1984), Science, 226, 956 - 959.

Lambros, C. and Bacchi, C.J., (1976), Biochem. Biophys. Res. Commun., 68(3), 658 - 667.

Lanham, S.M. and Godfrey, D.G., (1970), Exp. Parasitol., 28, 521 - 534.

Laveissiere, C. and Couret, D., (1980), Trans. Royal. Soc. Trop. Med. Hyg., 74, 264 - 265.

Lee, C-Y., Charles, D. and Bronson, D., (1979), J. Biol. Chem., 254(14), 6375 - 6381.

Libby, P., Alroy, J. and Pereira, M.E.A., (1986), J. Clin. Invest., 77(1), 127 - 135.

Long, C. (ed), (1961), "The Biochemist's Handbook", E. & F.N. Spon Ltd., London, 32.

Lowe, C.R., Harvey, M.J. and Dean, P.D.G., (1974a), Eur. J. Biochem., 41, 341 - 345.

Lowe, C.R., Harvey, M.J. and Dean, P.D.G., (1974b), Eur. J. Biochem., 41, 347 - 351.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., (1951), J. Biol. Chem., 193, 265 - 275.

Lumsden, W.H.R., (1972), Int. J. Parasitol., 2, 327 - 332.

Lumsden, W.H.R. and Hardy, G.J.C., (1965), "Nomenclature of Living

Parasite Material", Nature, 205, 1032.

Mackenzie, N.E., Hall, J.E., Seed, J.R. and Scott, A.I., (1982), Eur. J. Biochem., 121, 657 - 661.

Mackenzie, N.E., Hall, J.E., Flynn, I.W. and Scott, A.I., (1983), Bioscience Reports, 3, 141 - 151.

Mansfield, J.M. (ed), (1981), in "Parasitic Diseases: The Immunology", I, Marcel Dekker Inc., New York.

Markell, E.K. and Voge, M., (1981), "Medical Parasitology", 5th edn., W.B. Saunders Co., London.

McGinnis, J.F., (1983), Biochem. Biophys. Res. Commun., 116(2), 689 - 695.

McLaughlin, J., (1985), Mol. Biochem. Parasitol., 14(2), 219 - 230.

McLoughlin, D.J. and MacQuarrie, R., (1978), Biochim. Biophys. Acta, 527, 204 - 211.

Meshnik, S.R., (1984), in "Parasitic Diseases: The Chemotherapy", Mansfield, J.M. (ed), Marcel Dekker Inc., New York, 165 - 199.

Misset, O. and Opperdoes, F.R., (1984), Eur. J. Biochem., 144, 475 - 483.

Misset, O., Bos, O.J.M. and Opperdoes, F.R., (1986), Eur. J. Biochem., 157, 441 - 453.

Mollering, H., Wahlefeld, A.W. and Michal, G., (1974), Meth. Enz. Anal., 2nd edn., 1, 136 - 144.

Molyneux, D.H., (1982), in "Perspectives in Trypanosomiasis Research", Baker, J.R. (ed), Research Studies Press, John Wiley Ltd., Chichester.

Molyneux, D.H. and Ashford, R.W., (1983), "The Biology of Trypanosoma and Leishmania, Parasites of Man and Domestic Animals", Taylor and Francis, London.

Morrisey, J.H., (1981), Analyt. Biochem., 117, 307 - 310.

Newman, A.A., (1968), "Glycerol", Leonard Hill Books, London, 17.

Newton, B.A., (1968), Ann. Rev. Microbiol., 22, 109 - 130.

Nielsen, B.L. and Brown, L.R., (1984), Analyt. Biochem., 141, 311 - 315.

Niesel, D.W., Bewley, G.C., Miller, S.G., Armstorng, F.B. and Lee, C-Y., (1980), J. Biol. Chem., 255(9), 4073 - 4080.

Njogu, R.M., Whittaker, C.J. and Hill, G.C., (1980), Mol. Biochem.

Parasitol., 1, 13 - 29.

Nwagwu, M. and Opperdoes, F. R., (1982), Acta Tropica, 39
, 61 - 72.

Oduro, K.K., Flynn, I.W. and Bowman, I.B.R., (1980a), Exp. Parasitol., 50, 123 - 135.

Oduro, K.K., Bowman, I.B.R. and Flynn, I.W., (1980b), Exp. Parasitol., 50, 240 - 250.

Opperdoes, F.R., (1985), Brit. Med. Bull., 41(2), 130 - 136.

Opperdoes, F.R., Aarsen, P.N., van der Meer, C. and Borst, P. (1976a), Exp. Parasitol., 40, 198 - 205.

Opperdoes, F.R., Baudhuin, P., Coppens, I., de Roe, C., Edwards, S.W., Weijers, P.J. and Misset, O., (1984), J. Cell. Biol., 98, 1178 - 1184.

Opperdoes, F.R. and Borst, P., (1977), FEBS Lett., 80(2), 360 - 364.

Opperdoes, F.R., Borst, P., Bakker, S. and Leene, W., (1977), Eur. J. Biochem., 76, 29 - 39.

Opperdoes, F.R., Borst, P. and Fonck, K., (1976b), FEBS Lett., 62(2), 169 - 172.

Opperdoes, F.R. and Cottem, D., (1982), FEBS Lett., 143(1), 60 - 64.

Pace, G.W., Archer, M.C. and Tannenbaum, S.R., (1974), Analyt. Biochem., 60, 649 - 652.

Patthey, J-P. and Deshusses, J., (1987), FEBS Lett., 210(2), 137 - 141.

Peterson, G.L., (1979), Analyt. Biochem., 100, 201 - 220.

Polson, A., Potgieter, G.M., Largier, J.F., Mears, G.E.F. and Joubert, F.J., (1964), Biochim. Biophys. Acta, 82, 463 - 475.

Poltera, A.A., (1985), Brit. Med. Bull., 41(2), 169 - 174.

Raval,D.N. and Wolfe, R.G., (1962), Biochemistry, 1(2), 263 - 269.

Rée, G.H., (1985), Parasitol., 90, 617 - 620.

Reynolds, C.H., (1975), Biochem. Biophys. Res. Commun., 67(2), 538 - 543.

Reynolds, S.J., Yates, D. and Pogson, C.I., (1971), Biochem. J., 122, 285 - 297.

Rifkin, M.R., (1978), Proc. Natl. Acad. Sci. USA, 75, 3450 - 3454.

Ryley, J.F., (1955), Biochem. J., 59, 353 - 361.

Ryley, J.F., (1956), Biochem. J., 62, 215 - 222.

Ryley, J.F., (1962), Biochem. J., 85, 211 - 223.

Smyth, J.D., (1976), "Introduction to Animal Parasitology", 2nd edn., Hodder & Stoughton, London.

Srivastiva, H.K. and Bowman, I.B.R., (1971), Comp. Biochem. Physiol., 40(B), 973 - 981.

Tait, A., (1983), Parasitol., 86(4), 29 - 57.

Ukoli, F.M.A., (1984), "Introduction to Parasitology in Tropical Africa", John Wiley and Sons Ltd., Chichester.

van der Meer, C. and Zwart, D., (1980), in "The Host-Invader Interplay", van den Bossche, H. (ed), Elsevier/North-Holland Biomedical Press, Amsterdam.

van Meirvenne, N. and Le Ray, D., (1985), Brit. Med. Bull., 41(2), 156 - 161.

Vickerman, K., (1969), J. Cell Sci., 5, 163 - 193.

Vickerman, K., (1985), Brit. Med. Bull., 41(2), 105 - 114.

Vickerman, K., (1986), Nature, 322, 113 - 114.

Vickerman, K. and Cox, F.E.G., (1967), "The Protozoa", John Murray, London.

Visser, N. and Opperdoes, F.R., (1980), Eur. J. Biochem., 103, 623 - 632.

Visser, N., Opperdoes, F.R. and Borst, P., (1981), Eur. J. Biochem., 118, 521 - 526.

von Brand, T., (1966), "Biochemistry of Parasites", Academic Press, London.

Wakelin, D., (1984), "Immunity ot Parasites: How Animals Control Parasite Infections", Edward Arnold, London.

Walsh, D.A. and Sallach, H.J., (1965), Biochemistry, 4(6), 1076 - 1085.

Watson, D.H., Harvey, M.J. and Dean, P.D.G., (1978), 173, 591 - 596.

Wharton, C.W. and Eisenthal, R., (1981), "Molecular Enzymology", Blackie, London.

Whitfield, P.J., (1979), "The biology of Parasitism: an Introduction

to the Study of Associating Organisms", Edward Arnold, London.

World Health Organisation, (1983), "The Use of Essential Drugs", WHO Technical Report Series No. 685.

Volume 181, number 1 FEBS 2242 February 1985

The aerobic/anaerobic transition of glucose metabolism in *Trypanosoma brucei*

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Received 13 December 1984

The ratio of glycerol to pyruvate produced by *T. brucei* incubated with glucose at various oxygen tensions has been used as an index of the aerobic and anaerobic pathways of glucose metabolism. A minimal model is presented which fits the observed data. The value of the notional K of the aerobic/anaerobic transition from the model is close to that of the K_m of trypanosomal glycerophosphate oxidase. The anaerobic pathway appears to be almost completely inoperative at oxygen tensions in the range of those found in venous and arterial blood.

Trypanosoma brucei *Glucose* *Glycolysis* *Oxygen* *Glycerol* *Pyruvate*

1. INTRODUCTION

African trypanosomes of the *brucei* group undergo a complex life cycle involving the salivary glands and midgut of the insect vector and the bloodstream of the mammalian host. The bloodstream form of these trypanosomes has neither lipid nor carbohydrate reserves, nor does the mitochondrion possess a competent citric acid cycle. Glucose is the most important nutrient for these cells and the major and perhaps sole energy-yielding metabolic pathway is glycolysis (review [1]). Bloodstream trypanosomes contain no lactate dehydrogenase [2] and the NADH formed in the glyceraldehyde phosphate dehydrogenase step is reoxidized to NAD via a 3GP shunt [3] involving NAD-linked 3GP dehydrogenase which is localized in the glycosome [4] and mitochondrial 3GP oxidase [5].

Thus under aerobic conditions glucose is metabolized almost completely to pyruvate, approx. 2 mol pyruvate being produced per mol glucose [6–9]. Under anaerobic conditions or in the presence of an inhibitor of 3GP oxidase, such as salicylhydroxamic acid [8], glucose is metabolized to equimolar quantities of glycerol and pyruvate [6–9].

We wished to assess the relative importance of the aerobic and anaerobic pathways of glucose metabolism in trypanosomes in vivo; i.e., in the bloodstream of the mammalian host. However, the free oxygen concentrations of venous and arterial blood do not correspond to those of total anaerobiosis and aerobiosis. To learn more about the nature of the aerobic/anaerobic transition we investigated the dependence on O_2 concentration of the ratio of glycerol to pyruvate produced by these cells at oxygen tensions in the physiologically significant range which is intermediate to the extremes of full aerobiosis and anaerobiosis.

Abbreviations: 3GP, L-glycerol-3-phosphate; DHAP, dihydroxyactone phosphate; Po_2, partial pressure of oxygen

2. EXPERIMENTAL

Enzymes and cofactors were obtained from Sigma, London. All other chemicals were of Analar grade.

Cells of the long slender form of *T. brucei* were isolated from the blood of 250 g Wistar rats 71 h

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

after infection with 10^7 cells of strain MITat 1.1 (obtained from Dr H.P. Voorheis of Trinity College Dublin) by intraperitoneal injection. The cells were separated from blood components by centrifugation (600 × *g*) for 10 min at 4°C and further purified on a short column of DEAE cellulose in isotonic phosphate-buffered saline (pH 8.0) containing 10 mM glucose (Buffer A), as described by Lanham and Godfrey [10]. Cell counts were performed on a Neubauer haemocytometer. Glycerol and pyruvate were estimated in the same assay, from extent of NADH oxidation in the presence of lactate dehydrogenase, pyruvate kinase, phospho*enol*pyruvate and ATP. Glycerol kinase was added last for the determination of glycerol [11]. The assays were performed in triplicate and were standardized by using glycerol/pyruvate solutions of known concentration. Glucose was determined by using hexokinase and glucose-6-phosphate dehydrogenase as in [11].

Incubations at various oxygen tensions were carried out at 37°C in RPMI medium 1640 or in RPMI salts medium, initial pH 8.1, incorporating 150 mg% bovine serum albumin. These media have been shown to be capable of maintaining glycolysis and cell motility of *T. brucei* over the time periods used [9]. Seventy-five ml of the medium was equilibrated with the appropriate N_2/O_2 mixture in a 3-necked flask fitted with a gas inlet/outlet tube, probe type oxygen electrode (L.H. Fermentation, Model 507) and a rubber septum through which a large-bore syringe needle was inserted below the surface of the medium. The gas mixture, prehumidified by prior passage through water, was bubbled through the medium via a sintered tube at 200 ml/min. Oxygen concentration was monitored continuously and did not vary by more than ± 2 mmHg Po_2 during the incubations. Cells were introduced through the needle to give a final concentration of 2×10^7 cells/ml. At predetermined times 1-ml samples were withdrawn via the needle. A small amount was retained for cell count and the remainder quickly spun in a microfuge; this operation took less than 90 s. The cell-free supernatant was assayed for glycerol and pyruvate. In some experiments glucose was also assayed to confirm that the sum of glycerol and pyruvate could account for the glucose consumed. Metabolic competence of the cells in the withdrawn samples was confirmed by measuring the respiration rates in Buffer A using a Clark oxygen electrode.

3. RESULTS AND DISCUSSION

3.1. *Glycerol and pyruvate production*

Sample results for three oxygen tensions are shown in fig.1. It can be seen that the rate of production of glycerol plus pyruvate is about the same at all Po_2 values and that glycerol is produced at all oxygen tensions. Under anaerobic conditions glycerol and pyruvate are produced at roughly equal rates, glycerol production slightly exceeding that of pyruvate. At higher Po_2 values pyruvate is the major product but there is also a small but significant rate of glycerol production.

The ratio glycerol : pyruvate in the medium after 60 min incubation is plotted against Po_2 in fig.2, this ratio being a convenient way of expressing the relative involvement of the aerobic and anaerobic pathways of glucose metabolism. The ratio is independent of cell count and also of sampling time between 30 and 75 min. As seen in fig.2 the glycerol : pyruvate ratio remains constant at approx. 0.1 from a Po_2 of 160 mmHg down to below 40 mmHg from which it rises to a limiting value of approx. 1 under anaerobic conditions.

3.2. *Analysis of the aerobic/anaerobic transition*

At high oxygen tensions the 3GP shunt operates between the glycosome and the mitochondrion and provides a means for regenerating the DHAP reduced by NADH. Thus the equivalent of both triose phosphates can be converted to pyruvate resulting in 2 mol pyruvate per mol glucose. Under anaerobic conditions the 3GP shunt is inoperative. The DHAP reduced to 3GP cannot be regenerated and the 3GP is dephosphorylated and excreted from the cell as glycerol in a 1:1 ratio with pyruvate.

This scheme can be described by the following minimal model:

$$\text{glucose} + O_2 \longrightarrow 2\ \text{pyruvate} + H_2O \qquad (1)$$

$$\text{glucose} \longrightarrow \text{pyruvate} + \text{glycerol} \qquad (2)$$

where (1) and (2) represent the aerobic and anaerobic pathways, respectively.

Let V = rate of pyruvate production via pathway (1) under total aerobiosis, and v = rate of pyruvate

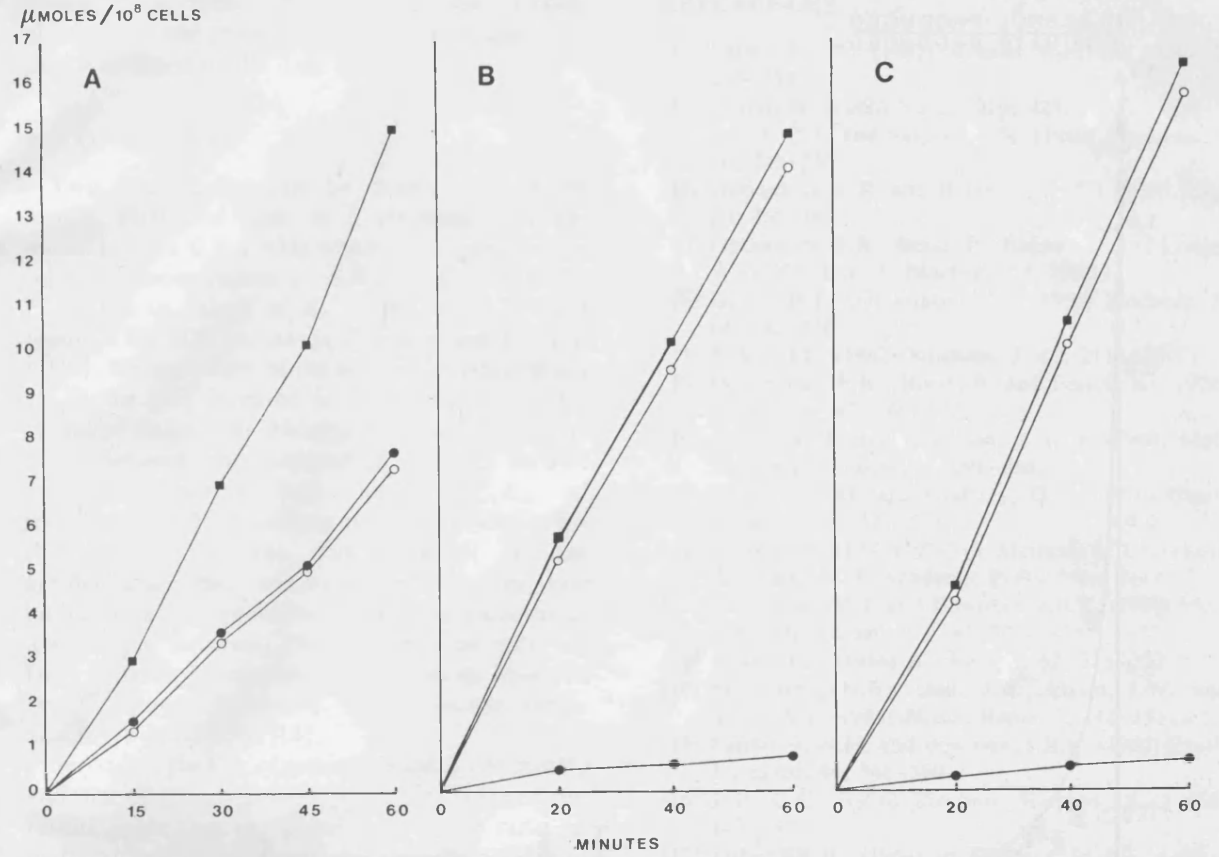

Fig.1. Glycerol and pyruvate production from *T. brucei*. For details see text. Po_2: 0 mmHg (A); 16 mmHg (B); 160 mmHg (C). (Glycerol) + (pyruvate) (■); (glycerol) (●); pyruvate (○).

production via pathway (1) under partial aerobiosis.

It is reported [8] and we have confirmed that bloodstream trypanosomes utilize glucose at about the same rate under aerobic or anaerobic conditions. This holds also at intermediate oxygen tensions. Thus $(V - v)/2$ = rate of glycerol (or pyruvate) production via pathway (2) at any level of aerobiosis.

As the total rate of production of glycerol plus pyruvate is constant it therefore follows that

$$\frac{(\text{glycerol})}{(\text{glycerol}) + (\text{pyruvate})} = \frac{V - v}{2V}$$

and

$$\frac{(\text{glycerol})}{(\text{pyruvate})} = \frac{V - v}{V + v} \qquad (3)$$

If we assume that the rate of pyruvate production via pathway (1) follows a Michaelian dependence on O_2 concentration:

$$v = \frac{V(O_2)}{K + (O_2)}$$

where K is a notional K_m of the aerobic pathway for oxygen, then by substitution into (3) we obtain

$$\frac{G}{P} = \frac{K}{K + 2(O_2)} \qquad (4)$$

Eqn 4 tends to zero at high O_2 concentrations and becomes unity when $(O_2) = 0$.

In fact we find that small but significant amounts of glycerol in excess of that predicted by theory are produced at all levels of aerobiosis, even when gassing with 95% oxygen. Glycerol : pyruvate ratios greater than one under anaerobic conditions

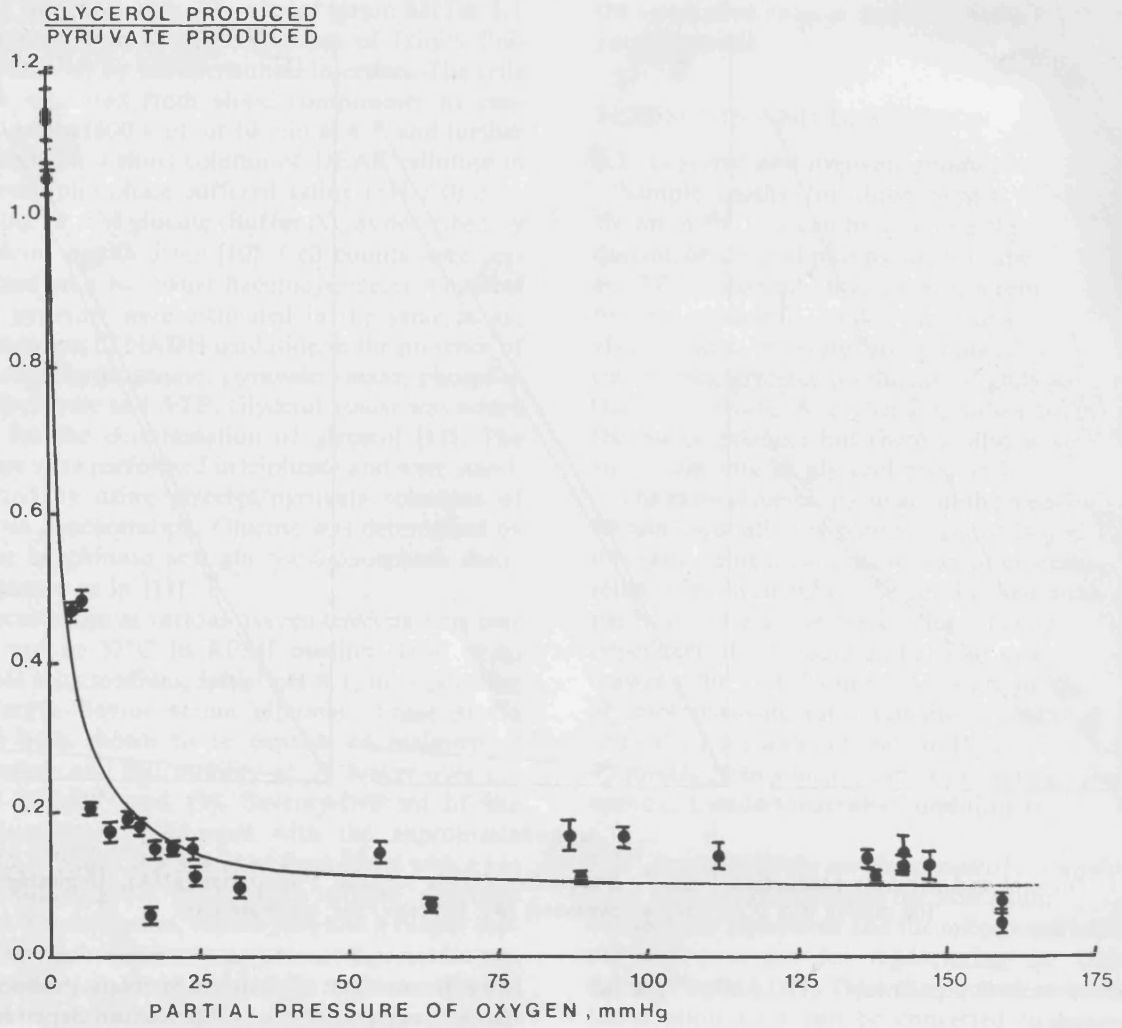

Fig.2. Ratio of glycerol:pyruvate concentrations in the medium of *T. brucei* incubated with glucose at various Po_2. The procedure is described in the text. The points are experimental, the bars representing standard errors of estimation. The curve is the best fit by least squares to the data of glycerol/pyruvate = $K/(K + 2(O_2)) + C$; $C = 0.09$, $K = 3.6 \pm 0.8$ mmHg.

reported by other workers [7,13,14] were consistent with our results. Under aerobic conditions glycerol:pyruvate ratios as low as 0.02 have been reported [6]; however, ratios as high as 0.24 and 0.4 were obtained by Ryley [7,13] for *T. rhodesiense* and *T. brucei*. Similarly, values of 0.11 and 0.1 were found by Grant and Fulton [6] and by Fairlamb and Bowman [15]. These latter workers ascribed the presence of glycerol in aerobic incubations to transient anaerobiosis experienced by the cells during workup but this is unlikely to be the case in the present study. Insufficient information is available to speculate profitably on the failure of the accepted pathways of glucose catabolism to account for all the glycerol found. Whatever the cause, we have corrected for this constant amount of glycerol by modifying eqn 4 to:

$$\frac{q}{p} = \frac{K}{K + 2(O_2)} + C$$

where C represents the effect of the 'aerobic glycerol' on the glycerol:pyruvate ratio; the equation was fitted to the data in fig.2.

4. CONCLUSION

Two conclusions can be drawn from these results. First, the value of K obtained from the model is 3.6 ± 0.8 mmHg which corresponds to an oxygen concentration of $5.0\ \mu$M. This may be compared to values of K_m in the range $2-8\ \mu$M reported for 3GP oxidase in *T. brucei* and *T. evansi* [16]. We are aware of the difficulties pointed out by Fisher [17] involved in interpretation of K_m values of intact cells. Nevertheless the close agreement between the notional K of the aerobic pathway reported here and the K_m of trypanosomal 3GP oxidase provides evidence for this enzyme as the control point in the aerobic/anaerobic transition and implies rate limitation at this point for the aerobic pathway at low oxygen tensions. This is consistent with the large increase in intracellular 3GP levels observed in *T. brucei* incubated with glucose under anaerobic conditions [18].

Secondly, the Po_2 of arterial blood is 100 mmHg and that of venous blood is 40 mmHg [19] and our results show that the glycerol:pyruvate ratio remains virtually constant in this range at a value of approx. 0.1. It thus appears that the anaerobic pathway of glucose metabolism of *T. brucei* has little if any physiological significance in the bloodstream of the mammalian host.

ACKNOWLEDGEMENTS

We thank Drs Paul Voorheis, Bernard Silverman, and Janet Pryke for their considerable help and advice, and the University of Bath for the award of a research studentship to A.P.

REFERENCES

[1] Fairlamb, A. (1982) Trends Biochem. Sci. 7, 249–253.
[2] Dixon, H. (1966) Nature 210, 428.
[3] Grant, P.T. and Sargent, J.R. (1960) Biochem. J. 76, 229–237.
[4] Opperdoes, F.R. and Borst, P. (1977) FEBS Lett. 80, 360–364.
[5] Opperdoes, F.R., Borst, P., Bakker, S. and Leene, W. (1977) Eur. J. Biochem. 76, 29–39.
[6] Grant, P.T. and Fulton, J.D. (1957) Biochem. J. 66, 242–250.
[7] Ryley, J.F. (1962) Biochem. J. 85, 211–250.
[8] Opperdoes, F.R., Borst, P. and Fonck, K. (1976) FEBS Lett. 62, 169–172.
[9] Brohn, F.H. and Clarkson, A.B. jr (1980) Mol. Biochem. Parasitol. 1, 291–305.
[10] Lanham, S.M. and Godfrey, D.G. (1970) Exptl. Parasitol. 28, 521–524.
[11] Bergmeyer, H.U. (1974) in: Methods of Enzymatic Analysis, vol.3, Academic Press, New York.
[12] Hammond, D.J. and Bowman, I.B.R. (1980) Mol. Biochem. Parasitol. 2, 63–75.
[13] Ryley, J.F. (1956) Biochem. J. 62, 215–222.
[14] Mackenzie, N.E., Hall, J.R., Flynn, I.W. and Scott, A.I. (1983) Biosci. Repts. 3, 141–151.
[15] Fairlamb, A.H. and Bowman, I.B.R. (1980) Exptl. Parasitol. 49, 366–380.
[16] Hill, G.C. (1976) Biochim. Biophys. Acta 456, 149–193.
[17] Fisher, R.B. (1964) in: Oxygen in the Animal Organism, pp.339–349, Pergamon Press, Oxford.
[18] Visser, N. and Opperdoes, F.R. (1980) Eur. J. Biochem. 103, 623–632.
[19] Cantarow, A. and Trumper, M. (1962) in: Clinical Biochemistry, p.350, W.B. Saunders, Philadelphia.